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A Microcolorimetric Method for the Determination of Quinic Acid and its Content in Flue-cured Tobacco

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Received, February 6, 1958

The colored compound produced by the reaction of periodate oxidation product of quinic acid with sodium nitroprusside and piperazine can be used as a quantitative measure of the quinic acid. The curve is linear for the acid examined in the range of 25 to 150μg of quinic acid per 1 ml. Hence, the reaction provides a convenient method for the determination of the micro quantities of quinic acid obtained from tobacco extracts fractionated by chromatography on paper or ion exchange-resin column. The content of quinic acid in flue-cured tobacco was 0.23 per cent to dry matter.

In a previous paper,[1] the author has reported that chlorogenic acid increased during flue-curing, and also that chlorogenic acid may be synthesized during flue-curing from quinic acid and caffeic acid; the latter may be derived from sedoheptulose through shikimic acid. Shikimic acid in flue-cured tobacco has been identified and determined as described in a previous paper.[2] Quinic acid in flue-cured tobacco is investigated in this paper.

Formerly, König and Dörr[3] found quinic acid from hydrolysis mixture of tobacco extracts. However, Schmuck and Piatnicki[4] were unable to detect free quinic acid in fermented Turkish Tobacco. Likewise, Dawson[5,6] has reported the absence of this compound in cured Connecticut cigar wrapper samples. More recently, Palmer[5,7] has demonstrated free quinic acid in mature, fresh cigar leaves and has shown that there is a loss of quinic acid during drying of the leaves at 80°. Possibly, this effect either through laboratory manipulation or by "natural" drying (curing), may have contributed to reported failures to detect free quinic acid in certain tobaccos.[5]

Quinic acid in other plant tissues,[8~13] has been found in a considerable amount. However, the determination of quinic acid has not been reported up to the present. Cartwright and Roberts found that the periodate oxidation product of quinic acid reacts with sodium nitroprusside and piperazine, giving an orange yellow color.[14] This reaction is specific to quinic, shikimic and dihydroshikimic acids.[15] The absorption spectrum of this orange yellow color assumed a maximum value at 390 mμ for quinic acid.

The author has now attempted a spectrophotometric determination of quinic acid, based on the reaction described above, and succeeded to identify and determine free quinic acid in flue-cured tobacco.

---

[1] M. Nagasawa, This Bulletin, **22**, 21 (1958).

[2] M. Nagasawa, This Bulletin, **22**, 174 (1958).

[3] P. König and W. Dörr, *Biochem. Z.*, **263**, 295 (1933).

[4] A. Schmuck and M. Piatnicki. *USSR State Inst. Tobacco Invest. Bull.*, **69**, 19 (1930) : *C. A.* **25**, 3124.

[5] R. L. Stedman, *Tobacco Sci.*, **1**, 155, pub. in *Tobacco*, 145, No. **15**, 22 (1957).

[6] R. F. Dawson, M. Solt and E. Wada, "Progress report on Cigar Manufactures Association Research Grant" (Columbia Univ., 1955).

[7] J. K. Palmer, *Science*, **126**, 504 (1957).

[8] A. C. Hulme, *J. Exp. Bot.*, **2**, 298 (1951).

[9] A. C. Hulme and T. Swain, *Nature*, **168**, 254 (1951).

[10] E. F. L. J. Anet and T. H. Reynolds, *Austral. J. Chem.*, **8**, 267 (1955).

[11] A. C. Hulme and A. Richardson, *J. Sci Food Agr.*, **5**, 221 (1954).

[12] A. Jermstadt, *Pharm. Acta Helv.* **25**, 209 (1950): *C. A.* **46**, 1726d.

[13] A. B. Swendsen, *Pharm. Acta Helv.*, **25**, 230 (1950): *C. A.* **46**, 1726d.

[14] R. A. Cartwright and E. A. H. Roberts, *Chem. & Ind.*, **1955**, 230.

[15] D. E. Hathway, *Biochem. J.*, **63**, 380 (1956).

EXPERIMENTAL

1. Preparation of Extracts. The same methanolic tobacco extracts as described in a previous paper[2] were used in this work.

2. Qualitative Separation and Identification. As described previously the methanolic extracts were investigated by paper chromatography using the following two solvents: (I) methylethylketone-cineole-formic acid-water (50 : 50 : 20 : 16),[8] (II) amylalcohol-5N formic acid (1 : 1).[16] Quinic acid was observed with R_F values in both solvents 0.09 and 0.08, respectively, and gave an orange-yellow color on spraying with periodate followed by sodium nitroprusside and piperazine as described by Cartwright and Roberts[12] (specific for quinic, shikimic and dihydroshikimic acids).[15] Identical R_F values (both alone and mixed) and an identical color were given for quinic acid detected and authentic acid in the above two solvent systems and reagent.

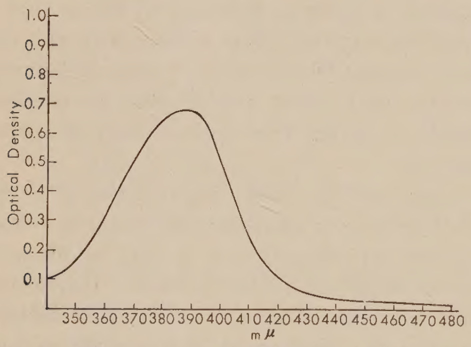

FIG. 1. Absorption of the Pigment derived from 100 μg Quinic Acid in 1 ml Water.

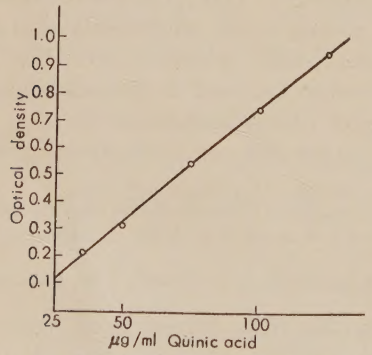

FIG. 2. Relationship between Color and Quinic Acid.

The eluates of the acid areas detected on filter paper sheets, developed using solvent (II), were developed again on Whatman filter paper using a phenol-water (3 : 1, w/v) mixture containing 0.9 per cent formic acid[17] and sprayed with the above reagent. Quinic acid was observed with a R_F value of 0.40, and identical R_F values and color were given for quinic acid detected and the authentic acid. However, dihydroshikimic acid could not be observed with a R_F value of 0.70 and a greenish color as described by Hathway.[15]

3. Quantitative Assay.

Reagent. The reagents of colorimetric determination of quinic acid are prepared as follows:

Periodate solution. Dissolve 160 mg of sodium periodate in 25 ml distilled water, prepare freshly daily.

Ethylene glycol solution. Dissolve 30 ml of ethylene glycol in 100 ml distilled water.

Carbonate buffer, pH 10.3. Mix one-part of 0.1 M sodium bicarbonate and 9 parts of 0.05 M sodium carbonate.

Sodium nitroprusside-piperazine solution. Dissolve each 500 mg of sodium nitroprusside and piperazine in 100 ml distilled water, and dilute to ten times, prepare freshly daily.

Ethanol, 95 per cent by volume.

Procedure. To 1 ml of the solution containing 25 to 120 μg of quinic acid add 1 ml of the periodate solution. Place the test tube in a thermostat of 30° for fifteen minutes. Then add 1 ml of the ethylene glycol solution, shake well, and maintain this in the above condition for five minutes. Remove the tube from the thermostat, add 1 ml of carbonate buffer and 1 ml of the sodium nitroprusside-piperazine solution. Heat the well-mixed solution for five minutes at 100° and cool for five minutes in running tap water. Make up the solution to a convenient volume with ethanol, usually taking 5 ml of ethanol, shake well, and determine the optical density, using a 1-cm cell on a Beckman DU spectrophotometer at 390 mμ. Read all tubes against blanks containing 1 ml of the periodate, the ethylene glycol, carbonate buffer, and the sodium nitroprusside-piperazine solutions subjected to the same procedure as above.

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[16] M. L. Buch, R. Montgomery and W. L. Porter, *Anal. Chem.*, **24**, 489 (1952).

[17] J. B. Stark, A. E. Goodban and H. S. Owerns, *Anal. Chem.*, **23**, 413 (1951).

The absorption spectrum, (measured a Hitachi recording spectrophotometer type EPS-2 Hitachi Ltd.), of the pigment derived from 100 μg quinic acid in 1 ml water are shown in Fig. 1. The absorption spectrum of this pigment assumed a maximum value at 390mμ as shown in Fig. 1. Various amounts containing about 25 to 120 μg of quinic acid in 1 ml were examined by the above procedure. The standard curve determined is shown in Fig. 2.

Application to Tobacco Extracts. The procedure described above was developed for the estimation of quinic acid in the tobacco extracts separated chromatographically on paper. One hundred and fifty microliters of the methanolic extracts (25 ml) were applied to Whatman No. 1 paper sheets. The chromatogram was run by the descending method using amylalcohol-5N formic acid (1 : 1) as the solvent. After about thirty hours, the strip was removed and dried in a hood. The areas detected by test spots on both sides of the filter paper sheets were cut out and eluted with water. The eluates were transferred quantitatively to a 10 ml volumetric flask and made to volume with water. One milliliter of the eluates was used for the estimation of quinic acid by the above method. The other eluates from the blank strip of Whatman No. 1 paper of an equal size were used for the blank solution.

Simultaneously, 100 μl of the solution containing 10 mg of quinic acid in 1 ml were applied to the same paper sheets, and recovery of quinic acid was obtained, upon being subjected to the same procedure as above.

RESULTS AND DISCUSSION

Palmer[5,7] has described that there is a loss of quinic acid in mature, fresh cigar leaves during drying of the leaves at 80°. Possibly this effect, either through laboratory manipulation or by natural drying (curing), may have contributed to reported failures to detect free quinic acid in certain tobaccos. However, it is clear from the results shown that quinic acid is present in flue-cured tobacco leaves. The content of quinic acid in flue-cured tobacco leaves was 0.23 per cent to dry matter. In regard to the determination of quinic acid, the author has encountered no report up to the present. By the above method, a series of concentrations ranging from about 6 to 200 μg of quinic acid in 1 ml water was examined, and at 390 mμ a linear relationship between color and the quantity of acid was found over the concentration 25 μg as shown in Fig. 2. Maximum color development occurred at pH 10 after five minutes heating at 100°. Therefore, the colored compound produced in the reaction of the periodate oxidation product of quinic acid with sodium nitroprusside and piperazine can be used as a quantitative measure for the quinic acid. The standards are run with each set determinations, since some fluctuation necessarily occurred in the optical densities.

For shikimic acid, the absorption spectrum of color presented by the above method, attained to a maximum value at 395 mμ, whereas, for quinic acid it was at 390 mμ. However, on the determination of shikimic acid, it is judged that the method described by Yoshida and Hasegawa[2] is more convenient, since their method is more sharp and simple.

Acknowledgement. The author wishes to thank Mrs. Taeko Taguchi for her assistance in carrying out the determination of quinic acid.

[Bull. Agr. Chem. Soc. Japan, Vol. 22, No. 4, p. 208~212, 1958]

The New Basic Triple Salt Containing Magnesium Hydroxide

Part I. The Quaternary System K_2SO_4-$MgSO_4$-$Mg(OH)_2$-H_2O at 100°

By Jiro SUGI and Michio NAKAYAMA
Central Research Institute, Japan Monopoly Corporation

Received February 6, 1958

The quaternary system K_2SO_4–$MgSO_4$–$Mg(OH)_2$–H_2O and the associated systems (a) K_2SO_4–$Mg(OH)_2$–H_2O and (b) $MgSO_4$–$Mg(OH)_2$–H_2O were investigated at 100°. Though isotherm (a) exhibited nothing new, isotherm (b) exhibited basic magnesium sulfate, $MgSO_4 \cdot 5Mg(OH)_2 \cdot 3H_2O$, as the solid phase. The solid phases of quaternary isotherm were the new basic triple salt $K_2SO_4 \cdot 2MgSO_4 \cdot Mg(OH)_2 \cdot 2H_2O$, langbeinite, basic magnesium sulfate, kieserite and potassium sulfate.

INTRODUCTION

In salt factories preparing salt from sea water, pH value of mother liquor at crystallization process is apt to lower to about 5.5. From the view point of preventing the corrosion of crystallizer, the authors once attempted to add magnesium hydroxide, which could easily be made being a base cheaply available in the factory, up to about 0.05 per cent in order to neutralize this lowered pH value. The result of this trial, however, ended in a failure, because the controlling effect of the pH value was rather small while on the contrary the mother liquor became very viscous. However, by investigating this phenomenon, the authors came to the following conclusions, namely, added magnesium hydroxide reacted with sodium, magnesium and sulfate ions in liquid and those elements formed themselves into a new basic triple salt, which was a fine needle crystal but grew radically from a grain of added magnesium hydroxide into something like a bar of chest nut, the mother liquor therefore becoming very viscous. The chemical formula of this basic triple salt was decided to be $Na_2SO_4 \cdot 2MgSO_4 \cdot Mg(OH)_2 \cdot 4H_2O$.[1)]

After further investigation of exchanging the composed ions of this triple salt to their intimate ions, the authors succeeded in exchanging sodium ion to potassium ion. Thus, a second new basic triple salt was discovered. The chemical formula of this second triple salt was decided to be $K_2SO_4 \cdot 2MgSO_4 \cdot Mg(OH)_2 \cdot 2H_2O$.

The authors have investigated various methods of separation of potassium dissolved in bittern for several years. In Japan, the method of utilization of potassium from bittern is essentially based upon the experiments of Hildebrand,[2)] that is, after the separation of carnallite, potassium chloride is obtained by hydrolyzing the separated carnallite. Following this method, however, potassium is given only as chloride. Research-works on the double decomposition of potassium chloride and sodium sulfate, which is the by-product of artificial silk manufacturing factories, have been performed to obtain potassium sulfate, but a method to separate the potassium directly as sulfate from bittern has never been established.

So, if the second triple salt were separated directly from natural bittern, it would be highly estimated in industrial interests, because, firstly, it would be able to be used as

---

[1)] M. Nakayama, *Bull. Soc. Salt Sci., Japan*, **10**, 188 (1956).

[2)] H. Hildebrand, *J. Ind. Eng. Chem.*, **10**, 96 (1918).

a new basic potassium-magnesium fertilizer which contain potassium and magnesium both as sulfate and moreover magnesium hydroxide, or secondly, potassium sulfate and magnesium sulfate heptahydrate would be obtained by hydrolyzing this.

From this point of view, the possibility of separating this triple salt directly from bittern was attempted and satisfactory results were obtained. Therefore, for the purpose of exploitation of this triple salt from bittern efficiently, the 100° isotherm for the four component system K_2SO_4–$MgSO_4$–$Mg(OH)_2$–H_2O and the associated systems K_2SO_4–$Mg(OH)_2$–H_2O and $MgSO_4$–$Mg(OH)_2$–H_2O were studied.

EXPERIMENTAL DETAILS

Definite amounts of purity tested potassium sulfate, magnesium sulfate, magnesium hydroxide and water were placed in Erlenmeyer flasks. The flasks were then immersed in an oil thermostat and sealed with a rubber plug after reaching the desired temperature. Liquid temperature was maintained at $100°\pm0.5°C$. Instead of stirring the liquid, the flasks were immersed in the thermostat for a period long enough (100–400 hours) and rotated from time to time by hand.

Sampling Liquid phase samples were filtered with a glassfilter, which was heated at about 100° by an electric heater surrounding it. To prevent evaporation of the filtered solution, weighed cold distilled water (about 15 g) was placed in a weighing tube in advance of filtration. About 15 g of the solution was usually taken for analysis.

Method of analysis Liquid phase samples were analyzed for potassium, magnesium, hydroxide and sulfate.

(a) **Potassium** In many cases potassium was estimated by differences of anions and cations, but in the case of involving important points such as invariant points, it was determined gravimetrically as potassium tetraphenyl boron.

(b) **Magnesium** was determined by N/20 E.D.T.A. titration, using Eriochrom Black T as the indicator.

(c) **Hydroxide** was determined by back titration with N/10 NaOH after the addition of excess N/10 H_2SO_4 to the sample.

(d) **Sulfate** The volumetric method was adopted. After the conversion of sulfate ion to sulfuric acid through cation exchange resin, it was determined by titration with N/10 NaOH using phenolphthalein as the indicator. As the cation exchange resin, Amberlite IR 120 crushed to 60–80 mesh was used. This method was not only speedy, but also completely accurate.

Solid phase samples were not analyzed chemically but examined by both power X-ray diffraction and petrographic microscope.

RESULTS AND DISCUSSION

System K_2SO_4–$Mg(OH)_2$–H_2O at 100° There has never been any reports concerning this system. Results obtained for this system are shown in Fig. 1. No double salt formation took place and nothing new resulted in this system.

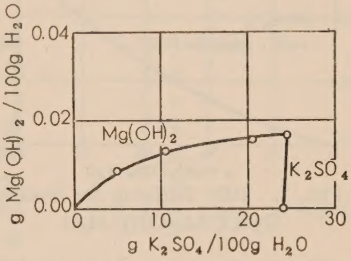

FIG. 1. 100° Isotherm for System K_2SO_4–$Mg(OH)_2$–H_2O.

System $MgSO_4$–$Mg(OH)_2$–H_2O at 100° Nothing has been reported on this system at 100°, but at other temperatures some reports such as those by M-L. Delyon,[3] L. Walter-Levy[4] and others[5,6] have appeared, that is, $MgSO_4\cdot 3MgO\cdot 11H_2O$ crystallized at 80° from the aqueous solution of magnesium sulfate between the concentration of 300 g/l and 500 g/l or $MgSO_4\cdot 5MgO\cdot 8H_2O$ crystallized at the boiling point from aqueous magnesium sulfate solution in the presence of a small amount of potassium bicarbonate or potassium carbonate. The Debye-Scherrer diagrams of these two double salts were investigated by Walter-Levy[4]. Further, $MgSO_4\cdot 5MgO\cdot 13H_2O$, $MgSO_4\cdot 3MgO\cdot 9H_2O$ appear in Gmelins Handbuch[7] with a question mark.

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[3] M-L. Delyon, *Bl. Soc. Chim.*, [5], **3**, 1811 (1936).
[4] L. Walter-Levy, *Ann. Chim.*, [11], **7**, 204 (1937).
[5] W. Feitknecht, *Helv. Chim. Acta*, **9**, 1813 (1926).
[6] G. Gloss, *Dissert. Berlin*, **1937**, 39.
[7] Gmelins Handbuch der anorganischen Chemie, Nr. 27, Mg B, s. 277, Verlag Chemie G. m. b. H., Berlin, (1930).

It is difficult to say from these reports that what kind of double salt takes place in this system at 100°. From our study on this system at 100°, the solid phase of all regions was the basic magnesium sulfate, $MgSO_4\cdot 5Mg(OH)_2\cdot 3H_2O$ and the solubility of magnesium hydroxide in this system are represented graphically in Fig. 2. The Debye-Scherrer diagram of this double salt essentially agreed with that of Walter-Levy.

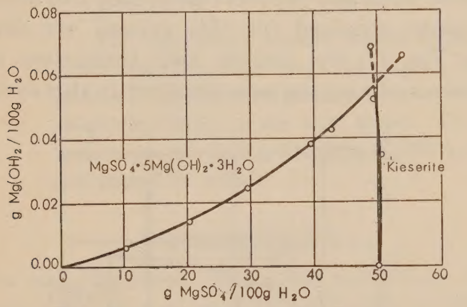

FIG. 2. 100° Isotherm for System $MgSO_4$–$Mg(OH)_2$–H_2O.

System K_2SO_4–$MgSO_4$–$Mg(OH)_2$–H_2O at 100° Nothing has yet been reported on this quaternary system, but for the ternary system K_2SO_4–$MgSO_4$–H_2O at 100°, two investigations were made by Starrs and Storch[8] in 1930 and Campbell and others[9] in 1934. The reported data of this ternary system, however, differ considerably from each other. In the course of the investigation of quaternary system, a choice was made between these two contradicted data.

Our results on this quaternary system are shown in Table I and represented graphically in Fig. 3, but as the concentration of dissolved magnesium hydroxide was so small, plotting is omitted in this figure. The large area enclosed in the curve BDTLNB indicate the crystallization field of the triple salt. The chemical formula of this triple salt is decided as $K_2SO_4\cdot 2MgSO_4\cdot Mg(OH)_2\cdot 2H_2O$.

Analytical data are:

Found: K, 15.33; Mg, 14.51; OH, 6.78; SO_4, 56.26; ignition loss (at 500°C), 10.66 %.

Calcd. for $K_2Mg_3(OH)_2(SO_4)_3\cdot 2H_2O$: K, 15.35; Mg, 14.32; OH, 6.68; SO_4, 56.58; ignition loss, 10.61 %.

The neighbouring area of this triple salt, MBDTK, are those of basic magnesium sulfate, $MgSO_4\cdot 5Mg(OH)_2\cdot 3H_2O$. In the region between points B and D, the authors found great interest, because if magnesium hydroxide was added to this region, an unknown substance which had an identified X-ray diffraction pattern appeared and if the triple salt was added to this region, it remained stable for more than 400 hours. The unknown crystal contains magnesium, hydroxide, sulfate and a small amount of potassium.

According to the less solubility of this triple

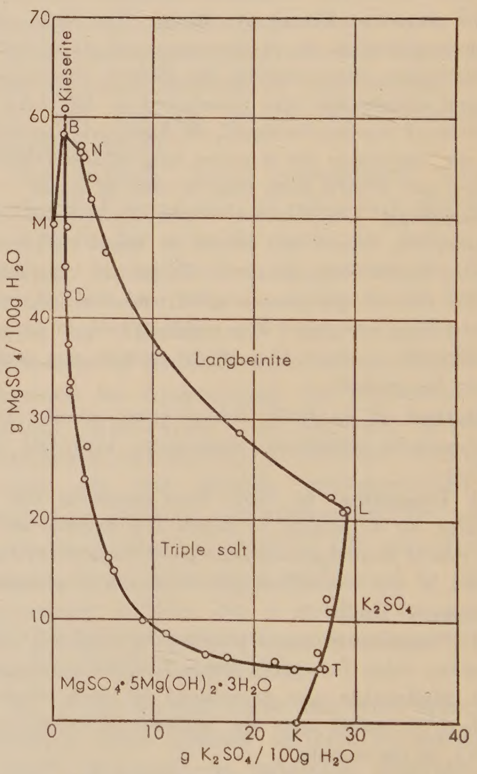

FIG. 3. 100° Isotherm for System K_2SO_4–$MgSO_4$–$Mg(OH)_2$–H_2O.

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[8] B. A. Starrs and H. H. Storch, *J. Phys. Chem.*, **34**, 2368 (1930).

[9] A. N. Campbell, K. W. Downes and C. S. Samis, *J.A.C.S.*, **56**, 2507 (1934).

TABLE I

DATA FOR 100° ISOTHERM FOR SYSTEM K_2SO_4–$MgSO_4$–$Mg(OH)_2$–H_2O

Points of Fig. 3	Composition of Liquid Phase Gram salts per 100 g H_2O			Agitation period Hrs.	Solid Phase Remarks*
	K_2SO_4	$MgSO_4$	$Mg(OH)_2$		
K	24.59	—	0.0167	96	Ks + $Mg(OH)_2$
T	26.52	5.317	0.0018	120	Ks + T. S. + B. M. S.
	26.90	5.295	0.0029	144	//
	(26.71	5.306	0.0024		// (mean))
	26.32	7.115	**	72	Ks + T. S.
	27.59	10.97	0.0071	96	//
	27.15	12.16	**	312	//
L	29.17	21.43	**	145	Ks + T. S. + Lang.
	28.88	21.09	**	145	//
	(29.02	21.26			// (mean))
	27.93	22.42	**	384	T. S. + Lang.
	18.63	28.88	**	72	//
	10.38	36.76	0.0028	72	//
	5.011	46.59	0.0044	145	//
	3.614	51.58		380	//
	3.789	54.09	0.0062	120	//
N	3.176	55.91	0.0073	405	T. S. + Lang. + Ms. 1
	2.725	57.16	0.0149	96	//
	2.702	56.46	0.0092	289	//
	(2.868	56.54	0.0105		// (mean))
	0.938	61.06	0.0669	115	T. S. + Ms. 1 + B. M. S. (supersaturation)
B	0.886	58.22	0.0661	312	T. S. + Ms. 1 + B. M. S.
M	—	49.36	0.0520	72	Ms. 1 + B. M. S.
	1.393	48.94	0.0232	168	T. S. + B. M. S.
	1.152	44.87	0.1108	145	//
D	1.456	42.44	0.0489	192	//
	1.599	37.44	0.0384	335	//
	1.872	33.62	0.0527	120	//
	1.868	32.91	0.0266	97	//
	3.453	27.19	0.0201	335	//
	3.130	24.29	0.0150	264	//
	5.733	16.31	0.0117	336	//
	6.141	15.03	0.0069	260	//
	8.883	10.20	0.0045	241	//
	11.29	8.832	0.0035	192	//
	15.22	6.606	0.0029	192	//
	17.38	6.478	0.0030	144	//
	22.10	6.262	0.0024	145	//
	23.89	5.205	0.0009	98	//

* Chemical formulae and names of solid phase are abbreviated as follows:
Ks = K_2SO_4, Ms. 1 = $MgSO_4 \cdot H_2O$, Lang. = Langbeinite,
B. M. S. = Basic magnesium sulfate $MgSO_4 \cdot 5Mg(OH)_2 \cdot 3H_2O$,
T. S. = Triple salt $K_2SO_4 \cdot 2MgSO_4 \cdot Mg(OH)_2 \cdot 2H_2O$.

** It was not determined because the solution was colorless in the presence of phenolphthalein.

FIG. 4. Microphotograph of Crystals of Basic Triple Salt ($K_2SO_4 \cdot 2MgSO_4 \cdot Mg(OH)_2 \cdot 2H_2O$) (×100)

salt, exploitation of potassium from aqueous solution of potassium sulfate and magnesium sulfate at 100° can be carried out more efficiently by way of this triple salt.

On the basis of these experimental data, it seems reasonable to conclude that the data reported by Starrs and Storch are better than those of Campbell and others, but in regions which contain much magnesium sulfate, their results seems to be erroneous.

Acknowledgement The authors are grateful to Prof. Dr. Shumpei Oka of Tokyo University for his kind advice and encouragement throughout the course of this study and also indebted to Mr. T. Matsuo and Miss S. Takabayashi for their assistance in carrying out this work.

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Studies on the Oxidation of Steroids by Fungi

Part I. The Steroid Oxidizing Property of *Corticium sasakii*

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As a result of seeking for suitable microorganisms which catalize the hydroxylation of steroids at C-11 position, *Corticium sasakii*, the fungus of sheath spot disease of rice, was found among the culture specimens of 28 genera of Basidiomycetes. This organism converted Reichstein's compound S to hydrocortisone, epi-hydrocortisone and 17α, 19-dihydroxydesoxycorticosterone.

Introduction of the hydroxyl group into the steroid nucleus is one of the most important processes in the synthesis of such medicines as cortisone, hydrocortisone, etc. Recently, it has become a common knowledge that microorganisms are available for this process, especially, for the introduction of oxygen into C-11 position. To search for a suitable microorganism to meet this purpose has been an important task in the field of applied microbiology. Microorganisms applied for this investigation have been mainly obtained from type cultures maintained in the known culture collections; i.e., the American Type Culture Collection, the Centraalbureau voor Schimmelcultures, Holland, etc. *Cunninghamella*,[1)]

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* The member of Research Laboratories attached to Takeda Pharmaceutical Industries Ltd.

[1)] F. R. Hanson, K. M. Mann, E. D. Nielson, H. V. Anderson, M. P. Brunner, J. N. Karnemaat, D. R. Colingsworth and W. J. Haines, *J. Amer. Chem. Soc.*, **75**, 5369 (1953).

Curvularia,[2] etc. have already been reported to be able to hydroxylate steroids at C–11β position, and *Aspergillus*,[3] *Rhizopus*,[4] etc. at C–11α position with high yields. In co-operation with Nishikawa* and his collaborators, we have conducted studies for the last few years on the steroid oxidizing ability of the fungi belonging to Ascomycetes, Basidiomycetes, and Fungi imperfecti preserved in our culture collection. As to Basidiomycetes, we investigated the type cultures in 28 genera and found several strains belonging to the genus Corticium to be able to introduce oxygen into C–11 position of steroids. *Corticium sasakii* (Shirai) Matsumoto, a species most useful for this purpose, is commonly known as the fungus causing the sheath spot disease of rice. This disease is widely found in Japan yearly in summer, and therefore, the isolation of this species is very easy. Experimental results with this fungus are presented below.

EXPERIMENTAL AND RESULTS

(1) Isolation of *Corticium sasakii* from natural source.

The sheath spot disease is easily distinguished from other sclerotial diseases of rice. The spots have ellipsoid or elongate shape, commonly 1 to 2 cm, but are often 4 to 5 cm in length. They are found on the outside of the sheath, and are at first greenish grey but turn greyish white with a blackish brown margin; colourless mycelium is seen on the outer or inner surface of the spots, sometimes with sclerotia.

For the isolation of this fungus, one of the sheath spots is cut off and soaked in a 0.1% solution of corrosive sublimate for about 30 to 60 minutes. After washing three times with sterilized water, the piece is put on a potato sucrose agar slant, and a pure culture is obtained from the leading edges of the mycelia of two days' growth.

On the slant, each hyphae is perpendicularly divergent and constricted at divaricated points. Aerial hypha are colourless and submerged mycelia are pale brownish to deep brown, 5 to 7.5μ (oftenly 9μ) in diameter. Sclerotia are white when young, then become brown or purple brown and their shape is globose or ovoid being more or less sharply defined but slightly flattened underneath. They vary in size, from 1 to 5mm broad, mostly 2 to 3 mm in diameter. Spores cannot be found.

(2) Cultivation of the fungus and oxidation of Reichstein's compound S

A quantity of 50 ml of K medium (Table I) in each 250 ml-shaking flask, was sterilized under 15 pounds steam pressure for 15 minutes. Each of the flasks was inoculated with a lump of vegetative mycelium of *C. sasakii*, which had been grown on a potato sucrose agar slant. The flask cultures were incubated at 28°C on a reciprocating shaker for 7 days. At the end of this period, 10 mg of 17α-hydroxydesoxycorticosterone (Reichstein's compound S on the market, which is abbreviated as S in our papers) was added as a sterile ethanolic solution to each flask and incubation was further continued for 48 hours at 28°C on a reciprocating shaker.

TABLE I

Sucrose	30 g	$MgSO_4 \cdot 7H_2O$	0.5 g
$NaNO_3$	2 g	Potato	50 g
K_2HPO_4	1 g	Wort	40 g
KCl	0.5 g	Tap water	1000 ml

pH ca 6.5, not adjusted

(3) Extraction and identification of steroids

As mentioned above, S was brought into contact with the vegetative mycelium in order to be converted. The conversion products thus obtained were scarcely contained in the mycelium but in the medium. After the culture broth was extracted by shaking for 20 to 30 minutes with 50 ml of ethyl acetate, the mycelium was separated from the broth filtrate through a Buchner funnel and extracted with acetone. The acetone extract was then evaporated and the residue was dissolved in ethyl acetate. The broth filtrate was also extracted with equal volumes of ethyl acetate two or three times. All the ethyl acetate solutions were combined, washed with a small volume of 1% NaOH solution, followed by distilled water, desiccated with Glauber's salt, and concentrated to dryness under reduced pressure in a stream of nitrogen.

The crude material thus obtained was a mixture consisting of the unchanged S and conversion products. These were separated from each other by a paper partition chromatography and the spots were compared with those of the authentic samples of hydrocortisone (it is abbreviated as F in our papers) and S.

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[2] G. M. Shull and D. A. Kita, *J. Amer. Chem. Soc.*, **77**, 763 (1955).

[3] J. Fried, R.W. Thoma, J.R. Gerke, J.E. Herz, M.N. Donin and D. Perlman, *J. Amer. Chem. Soc.*, **74**, 3962 (1952).

[4] D. H. Peterson and H. C. Murray, *J. Amer. Chem. Soc.*, **74**, 1871 (1952)

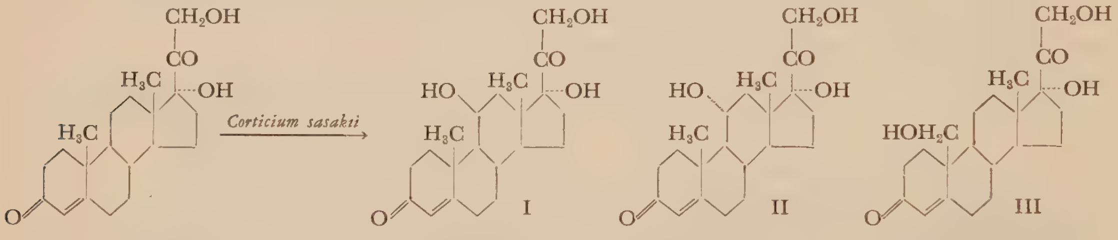
Since eminent studies on paper partition chromatography of steroids by Bush and Zaffaroni, many reports have been published in this field. We made use of a solvent system by Pechet5), which afforded a good result in the separation of the products. This system was composed of toluene · petroleum ether · ethanol · water (2:1:1:1). The R_F values of S and F obtained by this system were 0.57 and 0.10 respectively. The positions of the steroids separated on the paper chromatogram by this method were able to be detected by several colour reactions:6) triphenyltetrazolium chloride in alkaline solution (it is abbreviated as TPTZ in our papers) which is quite specific for a primary α-ketol group in the side-chain of steroids, antimony trichloride which gives a clear violet colour with F and a blue colour with S, and an iodine reagent which gives a blue colour with S although this blue colour disappears after a few minutes. With the R_F values of F and S, their colour reactions with these reagents were very available for distinguishing them from the other steroids. In addition to these colour reactions, the ultra-violet absorption at 240 mμ which is characteristic of Δ^4-3-ketosteroids, was adopted for the detection and quantitative determination of steroids. The five spots were found on the paper chromatogram through the above mentioned methods. (Table II)

TABLE II

No. of spot	R_F	Colour reaction		
		TPTZ	I_2	$SbCl_3$
I	0.10	+	—	violet
II	0.02	+	—	brown
III	0.03	+	—	blue
IV	0.34	+	—	brown
V	0.54	+	blue	blue

Spot V was identified as S not only by its R_F value and colour reactions but also by mixed chromatography with an authentic sample of S. Spot I was assumed to be F from its R_F value and colour reaction with antimony trichloride.

Among these conversion products, the compounds which were in accordance with the spots I, II, and III were isolated and identified.7) The infrared spectra of compound I and its monoacetate were identical with those of the authentic samples of hydrocortisone and its monoacetate respectively. Compound II and its diacetate coincided with epi-hydrocortisone and its diacetate in their physical constants and infrared spectra. Compound III was identified with 17α, 19-dihydroxy-11-desoxycorticosterone,8) while compound IV remained unascertained. It is noteworthy here that *Corticium sasakii* was the first microorganism which catalized C–19 hydroxylating reaction of steroids.



Reichstein's compound S

Hydrocortisone

Epi-hydrocortisone

17α,19-Dihydroxydesoxy-corticosterone

FIG. I

(4) Oxidation of S with the washed cell suspension

In the microbiological oxidation of steroids, two methods are generally employed. In one method, steroids as substrate are added to a culture broth and contacted with the growing cells (growing-cell method), while in the other, steroids are added into a buffer solution or tap water suspended with washed cells and are contacted with the resting cells (resting-cell method). These two methods do not always give the same effects, depending on the nature of fungi; i. e., *Rhizopus*, *Curvularia*, *Cunninghamella*, etc. We compared the latter method with the former in F production by *Corticium sasakii*.

These experiments were run in duplicate flasks and analytical data represent the average of the two flasks. According to the usual method, a strain of *Corticium*

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5) M. M. Pechet, *Science*, **121**, 39 (1955).

6) R. Neher and A. Wettstein, *Helv. Chim. Acta*, **34**, 2278 (1951).: R. B. Burton, A. Zaffaroni, and E. H. Keutmann, *J. Biol. Chem.*, **188**, 763 (1951).

7) T. Hasegawa, T. Takahashi, M. Nishikawa and H. Hagiwara, This Bulletin, **21**, 390 (1957).

8) M. Nishikawa and H. Hagiwara, *Chem. Pharm. Bull.*, **6**, 226, (1958).

sasakii was grown in 250 ml-flasks containing 50 ml of K medium on a reciprocating shaker for 7 days. Ten mg of S in 0.5 ml of ethanol was added to each culture, and incubation was continued for 48 hours. On the other hand, in the case of resting cell method the mycelium which was grown in the same way as in the growing cell method for 7 days was separated from the beer, washed with equal volumes of distilled water two times, suspended in 50 c.c. of distilled water containing 10 mg of S, and incubated for 16 hours on a reciprocating shaker. The extraction and the paper chromatography of the conversion products were the same as usual. The semi-quantitative method for the steroids separated on the paper chromatogram was as follows. Each spot of the steroids on the paper chromatogram detected by means of absorption of ultraviolet light was cut off carefully, and eluted with methanol. Absorption of the eluates was measured at 240 mμ with the use of a Beckmann Model DU spectrophotometer. The yields of conversion products were expressed as percentage based on the substrate added.

TABLE III

Method	epi-F 19-OH-S	F	Comp IV	S
Growing cells	32 %	33 %	7 %	—
Resting cells	33	28	9	—

Comparison of the resting-cell method with the growing-cell method is shown in Table III. When the above conditions were employed no remarkable difference between the results by these two methods could be observed with *Corticium sasakii*.

(5) Time of the addition of substrate

The growth rate of microorganisms is not the same depending on their natures and cultural conditions. The accumulation of F does not always increase with the growth of the microorganisms. For this reason, it is necessary to investigate the correlation of the yield of F to the age of growth of the fungus used. A quantity of 300 ml of the K medium in each of the 20 shaking flasks was inoculated with 30 ml of the four day-old culture of *Corticium sasakii*. These cultures were then divided into two groups. One group was used for the growing cell method and the incubation period after the addition of S was 48 hours. The other group was for the resting-cell method and the incubation period was 16 hours.

The relation of the incubation period before the addition of S and the weight of the mycelium to the yield of F is shown in Table IV. (The analytical data shows the average of the two flasks.) The results of this experiment indicate that the enzymatic activities of this fungus in F production are stable for 12 days, and that the yield of F seems to be proportional to the cell growth.

TABLE IV

Incubation period	Weight of damp mycelium	F from 50mg S by growing cells	F from 100mg S by resting cells
2 days	28 gr	24 %	25 %
4	35	28	30
5	—	33	—
6	46	32	32
7	—	33	—
9	37	—	30
12	37	—	29

(6) The decrease of F during incubation

Application of the quantitative method which comprises measuring of ultraviolet absorption at 240 mμ is limited to the steroids containing the Δ^4-3-ketone-structure. From the results of the studies conducted by many workers, this Δ^4-3-ketone structure of steroids is known to be attacked by many microorganisms. So, when the structure is changed, this application already becomes impossible. It is naturally considered that *Corticium sasakii* may also transform F at the Δ^4-3-ketone structure, and it is an important problem from the industrial point of view to observe to what extent the produced F will be further transformed by this fungus during incubation.

Ten mg of S or F was added as fine powder to each flask containing a suspension of 2 g of damp mycelium in 50 ml of tap water, and then contacted with the mycelium on a reciprocating shaker at 28 C. The results are shown in Fig. 2. Compound S was completely converted at 16 hours. While the recoveries of F at 16 and 24 incubation-hours were 82 and 60 percent of that at zero hour, respectively and no further

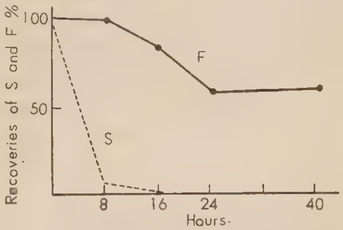

FIG. 2. Degradiation of F and S in Cell Suspension

TABLE V

Carbon compound	pH change during growth		Damp mycelium of 7 days' growth	pH change during oxidation		Yield of F (%)	S remained (%)
	Initial pH	Final pH		Initial pH	Final pH		
sucrose	6.5	7.0	100	7.0	6.6	23	0
glucose	6.5	7.0	80	7.0	6.2	16	0
fructose	6.5	5.6	17	5.6	5.8	22	27
maltose	6.5	7.2	67	7.2	7.0	25	0
lactose	6.5	5.8	15	5.8	5.8	18	28
raffinose	6.5	7.4	56	7.4	7.2	26	0
mannitol	6.5	7.0	47	7.0	7.2	28	0
sorbitol	6.5	7.5	52	7.5	7.3	27	0
soluble starch	6.5	7.0	117	7.0	7.2	26	0
potato starch	6.5	7.0	100	7.0	7.0	26	0
wheat strach	6.5	7.0	108	7.0	7.0	30	0

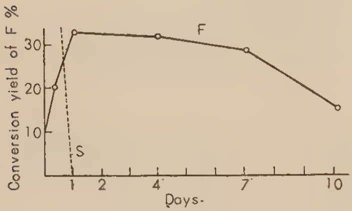

FIG. 3. Stability of F produced from S in the Culture Broth.

depression of recovery of F was found in the period from 24 to 40 hours. From this it may be considered that after 24 hours, the activity of the mycelium would be almost lost. The results obtained by the growing-cell method are shown in Fig. 3. An amount of 300 ml of the K medium was inoculated and incubated on a reciprocating shaker as before. After 2 days' incubation at 28°C, 50mg of S in 6 ml of ethanol was added to each flask and incubation was continued. The plot of the yield of F showed a curve descending slowly with incubation time. In spite of the sufficient supply of the fungus mycelium against the weight of substrate (see Table IV) the decrease of F in Fig. 3 was much slower than that in Fig. 2. The degradation of F may be considered to be influenced by other oxidation products of S and—or by the remaining nutrients in the medium. From these results, it was also found that there was a remarkable difference in the conversion rate between F and S. This brings out an important fact that the chemical structure of steroidal substrates exerts a direct influence upon the transformation of steroids in the microbiological method.

(7) Selection of the carbon source as a component of the medium.

The utilization of a fungus as a catalyser for steroid transformation requires a suitable medium for its growth and adequate contact of the mycelium with the substrate. For this reason, various other sugars added to K medium instead of sucrose were examined regarding their influences on the growth of fungus and on the transformation of S. The concentration of sugars in each medium was 3%.

A portion of 1 c.c. of a four-day old seed culture was inoculated in 50 c.c. of medium in each shaking flask. After 7 day's incubation, 10 mg of S in 1 c.c. of ethanol was added and incubation was continued for 48 hours. Then each broth was extracted with ethyl acetate. The mycelium produced in each flask was dried up at 100 C. 30 min., and weighed. The results are given as the percentage based on the weight of the mycelium produced in the sucrose medium (Table V).

Starches, sucrose and glucose were suitable for growth of this fungus and the transformation of S, but the accumulation of F did not always correspond to growth. This seems to be due to the high potencies of the cells in glucose and sucrose media, in which F might be further converted. We also observed that during the growth of this fungus, the change of pH of the media are very small, regardless of the kind of sugars.

DISCUSSION

The fungus described above, first found on the leaves of camphor trees by Sasaki was named *Hypochnus sasakii* by Shirai in 1906.9) Seven years later, Sawada10) stated that this

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9) M. Shirai, *Bot. Mag. Tokyo*, **20**, 319 (1906).

10) K. Sawada, *Formosan Agr. Exp. Sta. Spec. Bull.*, **4**, 80 (1912).

fungus was identical with *Sclerotium irregulare* Miyake on rice plants. In 1934, Matsumoto[11] compared it with *Rhizoctonia solani* Kuhn (=Corticium vagum Berk. & Curt.) and determined the taxonomical situation of this fungus to be *Corticium.* In 1953, Exner[12] shifted this situation to the genus *Pellicularia*, the name of which has, however, rarely been used by European taxonomists. Besides rice, this fungus attacks a large number of plants and has not been found usable to any extent. As for the oxidation of steroids by the fungus, one of its remarkable properties is to oxidize steroids to give both 11α- and 11β-hydroxysteroids. This property has not been reported except in *Absidia* species by Schmidt-Thome.*[13] It is very interesting to research about the relations which exist between the enzymatic properties producing both of these epimers.

It was observed that when pure hydrocortisone was added in the mycelium suspension, its recovery decreased to 60% after 24 hours, but when it was produced from S in the cultural broth, its decomposition was more moderate. Besides the foregoing facts, the influences of the structure of steroids and of the carbohydrates added in the culture medium were also observed. Generally speaking, in the microbiological conversion of steroids the conversion rate to their corresponding derivatives was found to be dependent upon many factors, such as the structure of steroids, nature of microorganisms, medium, aeration, temperature, etc. For the purpose of improving the yield of a certain steroid in aim such as hydrocortisone, these factors should be well controlled.

SUMMARY

1) The microbiological transformation of 17α-hydroxydesoxycorticosterone to hydrocortisone, epi-hydrocortisone, 17α, 19-dihydroxydesoxycorticosterone and minor unknown products by *Corticium sasakii* (Shirai) Matsumoto, has been demonstrated.

2) The above hydroxy-derivatives of the steroid were produced to the same degree by the resting-cell method as by the growing-cell method.

3) The steroid oxidizing activity of this fungus was stable and parallel with the weight of the mycelium.

4) As one of the medium components, several sugars were examined, starches, sucrose and glucose being found suitable for fungus growth. During incubation the hydrogen-ion concentration of the medium hardly shifted, regardless of the kind of sugars.

Acknowledgement We wish to express our sincerest thanks to Dr. K. Sato, Institute for Fermentation, to Dr. T. Matsukawa, Research Laboratories attached to Takeda Pharmaceutical Industries Ltd. and to Dr. K. Sakaguchi, University of Tokyo for their guidance and encouragement throughout this work. We heartily appreciate the valuable advice of Dr. Y. Asahina, Dr. S. Kuwada, and Dr. S. Akai.

Our acknowledgements are also expressed to Dr. M. Nishikawa and Mr. H. Hagiwara for their helpful advice.

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* Recently, Greenspan et al. have lightly touched upon the fact that some species of Rhizoctonia could hydroxylate steroids at C-11α and C-11β positions (*J. Amer. Chem. Soc.* **79**, 3922, 1957). Although details have not been mentioned in their communication, it is quite possible that this culture may be closely related to the cultures we had found. Our results were filed as a patent application in November of 1956).

11) T. Matsumoto, *Trans. Sapporo Nat. Hist. Soc.*, **13**, 115 (1934).

12) B. Exner, *Mycologia*, **45**, 698 (1953).

13) J. Schmidt-Thome, *Angew. Chem.*, **69**, 238 (1957).

[Bull. Agr. Chem. Soc. Japan, Vol. 22, No. 4, p. 218~227, 1958]

Studies on Amino Acids. XI.

Studies on the Enzymatic Resolution (IX): Acylase Activity in Yeast*

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Received March 10, 1958

As a part of the studies intending to clarify biological significance of the presence of acylase, enzymatic activity which hydrolyzes acyl amino acids, its activity in yeast was investigated. As a results, the occurrence in yeast of acylase activity was confirmed for the first time.

In order to study the enzymatic properties of this acylase activity, experiments were carried out with the enzyme preparation from brewer's yeast. As a result of the investigation, yeast acylase was found to be able to hydrolyze a number of acyl amino acids, of these chloroacetyl derivatives especially readily, as in the case of previously studied acylase activity in other sources such as mold acylase. Several observations on the influence of metal ions and inhibitors, optical specificity and others were also presented.

On the acylase activity, that is, hydrolytic activity towards acyl amino acids, Greenstein and his co-workers have made extensive studies with hog kidney acylase, by applying the enzyme for the optical resolution of amino acids.

The authors who have been studying on acylase activity in molds have reported on the practical resolution of amino acids.[1~6] Through these studies, the authors were interested in the distribution of acylase activity in various organisms and intended to clarify the biological significance of the activity. For this purpose, in succession to the previous studies on the acylase activity in *Escherichease*,[7] the authors have preformed experiments on the acylase preparation from yeast, for no available report has appeared on this acylase activity in yeast.

MATERIALS AND METHODS

Compounds Used—The preparation of the amino acid derivatives was reported by the authors in previous papers.

Enzyme Source—Brewer's yeast employed for this experiment was generously supplied by Dr. Y. Kuroiwa, Amagasaki Laboratory, Kirin Brewing Co. The supplied yeast cells were washed with water and collected by centrifugation before they were used as enzyme source.

Extraction of Acylase Activity from Yeast—On the extraction of various enzymes from yeast cells, a number of procedures have been reported. In order to find out an effective extraction method of acylase activity, the following procedures were investigated for comparison.

1. Grinding

Yeast (10 g) was ground with a little amount of quartz sand in a mortar and liquefied. After 30minutes,

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[1] S. Yamada, I. Chibata, and S. Yamada, *J. Pharm. Soc. Japan*, **75**, 113 (1955).

[2] I. Chibata, S. Yamada, and S. Yamada, This Bulletin, **20**, 174 (1956).

[3] I. Chibata, A. Watanabe, and S. Yamada, This Bulletin, **21**, 291 (1957).

[4] I. Chibata, A. Watanabe, and S. Yamada, This Bulletin, **21**, 296 (1957).

[5] I. Chibata, T. Ishikawa, and S. Yamada, This Bulletin, **21**, 300 (1957).

[6] I. Chibata, T. Ishikawa, and S. Yamada, This Bulletin, **21**, 304 (1957).

[7] I. Chibata, M. Kisumi, and S. Yamada, This Bulletin, **22**, 24 (1958).

M/15 phosphate buffer (10 ml) of pH 6.0 was added, and the mixture was kept for 12 hours and 72 hours at 5°. The supernatant solution obtained by centrifugation was filtered with Celite, and the filtrate was used as enzyme extracts.

2. Autolysis with Solvents

i) *With toluene*—To the yeast (10 g), water (10 ml) and a little amount of toluene were added. The mixture was allowed to stand at 37° for 24 hours with adjusting the pH to 6.0. The extracts were prepared as the same way as in the previous case.

ii) *With ethyl acetate*—When ethyl acetate (1 ml) was mixed with the yeast (10 g), liquefication occurred within a few minutes. After 15 minutes, water (10ml) was added, and the mixture was kept at 10° and 37° for 24 hours and 72 hours along with adjustment of the pH of the mixture to 6.0 and 7.0. The extracts were prepared as in the above case.

iii) *With chloroform*—The extracts were prepared with autolysis employing chloroform instead of ethyl acetate of the previous procedure. In this case, liquefication occurred within 5 minutes.

3. Extraction from Dried or Frozen Cells

i) *Air drying* (grinding with ball-mill)—Air-dried yeast was milled in a ball-mill for 10 hours at room temperature. The resulting powder (corresponding to 10 g of yeast) was extracted with M/15 phosphate buffer (pH 6.0) for 2 hours at 10° and 37°.

ii) *Acetone drying*—The yeast (80 g) suspended in water (70 ml) was added to cooled acetone (3 l) at −10° under vigorous stirring. After a brief stirring the cells were allowed to settle, and the precipitate was filtered and washed with acetone. Evaporation of the solvent gave an acetone-dried preparation (18.7 g).

A portion of this acetone powder was extracted with M/15 phosphate buffer (pH 6.0) for 2 hours with stirring at 10°. Another portion of the acetone-dried preparation was ground with quartz sand and the phosphate buffer was added. The mixture was kept for 15 hours at 5° and extracted.

iii) *Freezing and thawing*—The yeast (10 g) was freezed by dry-ice and ground in a mortar. After thawing, freezing and grinding were repeated. The thawed mixture were extracted with M/15 phosphate buffer (pH 6.0, 10 ml) by standing for 24 hours and 72 hours at 5°.

Throughout the above mentioned procedures, the yeast was extracted with the same amount of phosphate

TABLE I
ON THE EXTRACTION OF ACTIVITY FROM YEAST CELLS

		Procedure	pH	temperature (°C)	hours	increased O. D.
1		Grinding with quartz sand	6.0	5	12	118
		〃	6.0	5	72	259
2	i)	Autolysis with toluene	6.0	37	24	98
	ii)	Autolysis with ethyl acetate	7.0	37	24	30
		〃	6.0	10	24	101
		〃	7.0	10	24	100
		〃	7.0	37	72	79
		〃	6.0	10	72	99
		〃	7.0	10	72	109
	iii)	Autolysis with chloroform	7.0	37	24	21
		〃	6.0	10	24	117
		〃	7.0	37	72	286
		〃	6.0	10	72	279
3	i)	Air drying and grinding with ball will	6.0	37	2	385
		〃	6.0	10	2	355
	ii)	Acetone drying	6.0	10	2	66
		〃 (with grinding)	6.0	5	15	270
	iii)	Freezing and thawing	6.0	5	24	346
		〃	6.0	5	72	335

The incubation mixture contained 1 ml of 0.05M chloroacetyl-DL-methionine solution, 1 ml of ten-times diluted extracts and 1 ml of M/15 phosphate buffer (pH 6.0). The mixture was incubated at 37° for 24 hours. The liberated amino acid was determined by colorimetric ninhydrin method and the extent of reaction was expressed by increased O. D. for the comparison of extraction procedure.

buffer or water. The obtained extracts were diluted ten-times by volume and their hydrolytic activity was assayed by employing chloroacetylmethionine as the substrate. The results obtained are shown in Table I.

As indicated in the Table, grinding of air-dried yeast in a ball-mill, extraction by freezing and thawing, and autolysis by chloroform were found to be effective extraction procedures.

Enzyme Preparation—As the extraction procedure of acylase activity from yeast cells was investigated, preliminary experiments of purification of acylase activity from these extracts obtained by the above procedures, were performed for the purpose of obtaining a stable enzyme preparation.

Although the extracts obtained by freezing and thawing procedure and by ball-mill procedure showed high activity, these extracts were not so suitable for purification as the obstructive turbidity and viscosity. So, by employing the procedure of plasmolysis with chloroform, more suitable conditions for the enzyme preparation were investigated.

The yeast (20 g) was mixed with chloroform (2 ml) and liquified. The mixture was extracted with M/30 phosphate buffer (pH 6.0, 20 ml) or with 0.4 saturated aqueous ammonium sulfate (pH 6.0, 20ml) by keeping for 96 hours at 5° along with adjusting the pH to 6.0. In the other run, after liquefication, the same amount of water was added, stirred for 1 hour, and colored initial autolysate was removed by centrifugation. To the precipitate, chloroform (1 ml) and 0.4 saturated aqueous ammonium sulfate (pH 6.0, 20 ml) were added and extracted just as described in the above case. Activity of these extracts was assayed for comparison after they were dialyzed against chilled water under the some conditions.

As indicated in Table II, extraction with aqueous ammonium sulfate was much more effective than ordinary extraction with phosphate buffer, especially the process removing the colored initial autolysate gave a clear extract, which is suitable for enzyme preparation. So with this procedure, preparation of yeast acylase was carried out as follows: Brewer's yeast (1 kg) was mixed with chloroform (100 ml) for liquefication. After it was liquified, water (1 l) was added and the mixture was kept for 1 hour at 10° with occasional stirring. A colored supernatant solution was removed by centrifugation. To the separated precipitate, chloroform (50 ml) and 0.4 saturated aqueous ammonium sulfate (pH 6.0, 1 l) were added. For further autolysis, the mixture was allowed to stand for 96 hours at 10° with occasional adjustment of the pH to 6.0. Centrifugation gave a clear supernatant solution (1050 ml), which was used as the extract (1-C in Table II). To this extract, ammonium sulfate was added to 0.75 saturation at pH 6.0. The resulting precipitate was collected and dissolved in M/30 phosphate buffer (pH 6.0, 500 ml). The solution obtained was treated with ammonium sulfate, the precipitate resulting from 0.45 saturation was removed by centrifugation and discarded. The supernatant solution was brought up to 0.7 saturation with ammonium sulfate, and the resulting precipitate was collected with centrifugation. Then the precipitate was dissolved in M/30 phosphate buffer (pH 6.0) and dialyzed against chilled water for 2 days. The precipitate formed during dialysis was removed by centrifugation. Then, to the supernatant solution (265 ml, 2 in Table II), acetone (33 % by volume), previously chilled to −5°, was added with stirring. The precipitate was collected by centrifugation and dissolved in M/30 phosphate buffer (pH 6.0). A small amount of insoluble material was removed by centrifugation, and the clear supernatant solution was lyophylyzed. The obtained white powder weighed 4.6 g (3 in Table II), its yield of total activity being

TABLE II
EXTRACTION AND PURIFICATION OF YEAST ACYLASE

		Procedures	Acylase activity μM/hr/Prot. N mg	Total activity μM
Extraction Process	1-a	Phosphate buffer	22.2	992
	1-b	0.4 saturated ammonium sulfate solution	45.0	2480
	1-c	The same as 1-b except removing initial autolysate	52.3	2453
Purification Process	1-c	The extracts Prepared by 1-c	58.6	124200
	2	Ammonium Sulfate fraction and dialysis	75.2	113000
	3	Acetone fractionation and lyophylization	235.0	73000

The incubation mixture contained 1 ml of 0.025 M chloroacetyl-L-methionine solution, 1 ml of properly diluted enzyme solution and 1 ml of M/15 phosphate buffer (pH 6.0). The mixture was incubated at 37° for 2 hours. The liberated amino acid was determined by cololimetric ninhydrin method.

59% of the extract.

This preparation was stable for at least several months in a desicator kept in a cool place.

Standard Enzyme Reaction—Enzyme reaction was carried out by incubating digests composed of 1 ml of 0.025 M substrate solution (0.05 M for racemic compounds), 1 ml of enzyme solution and 1 ml of M/15 phosphate buffer (at appropriate pH, described in Table III) at 37° for 2 hours, unless otherwise stated. Liberated amino acids were measured by the colorimetric ninhydrin method, and expressed by per cent hydrolysis of the susceptible L-isomers. (The rate of hydrolysis was determined from the splitting of the susceptible substrate which was between 10 to 30 per cent).

For example, the effect of enzyme concentration on the rate of hydrolysis of acetyl-DL-methionine under standard conditions, is shown in Fig. 1.

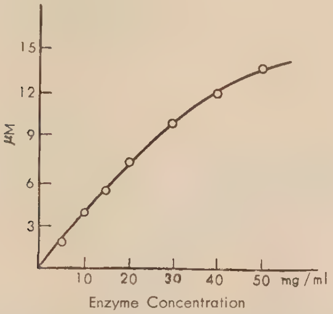

FIG. 1. Effect of Enzyme Concentration on Reaction Rate.

The mixture containing varying amount of enzyme and acetyl-DL-methionine was incubated at pH 6.0 for 2 hours under standard conditions.

RESULTS

Effect of pH and Buffers In a previous paper6), the authors observed that the optimal pH towards acetyl and chloroacetyl derivatives differed in case of fungal acylase preparation. Then, the effect of pH on the hydrolytic rate of various acyl acids by the yeast acylase preparation was studied and the optimal pH was determined. The results obtained are summarized in Table III.

TABLE III
OPTIMAL pH FOR THE HYDROLYSIS OF ACYL AMINO ACIDS

Acyl derivs.	Acetyl	Chloroacetyl
Alanine	6.5	6.5
Leucine	6.5	6.0
Methionine	6.3	6.0
Glutamic acid	5.0	5.0
Aspartic acid	5.5	5.0
ε-Benzoyllysine	6.5	6.5
Tryptophan	7.0	6.5
Phenylalanine	6.5	6.3
Tyrosine		6.5
Glycylglycine	7.8	
Glycylmethionine	7.5	

The reaction was carried out under standard conditions.

Although pH optima for the acyl derivatives of monoaminodicarboxylic acids such as aspartic acid and glutamic acid seemed to be on the acidic-side, optimal pH of other acyl amino acids were around 6.0~6.5, no appricible difference of optimal pH between acetyl and chloroacetyl derivatives being detected.

Besides acyl amino acids, optimal pH for the hydrolsis of dipeptides such as glycylglycine and glycylmethionine by this yeast preparation was also studied and it was found to be on the alkaline-side.

For the purpose of investigation of the influence of buffers, a number of other buffers, including Walpole acetate buffer, Michaelis Veronal buffer, and Sörensen citrate buffer, were tested as substitutes for Sörensen phosphate buffer. Acetylmethionine, chloroacetylmethionine, chloroacetylphenylalanine and chloroacetylglutamic acid were employed as the substrates, and conditions were standard except for the buffers. The pH-activity curves obtained are presented in Fig. 2.

A different pH optimum was found for each of the buffers, as well as a difference in the amount of hydrolysis took place. Veronal buffer permitted highest reaction rate so far as the acyl amino acids employed in this experiment, and citrate buffer was apparently inhibitory to the acylase activity.

In the case of Veronal buffer, optimal pH appreciably shifted to the acidic-side and the highest activity for the hydrolysis of acyl derivatives of neutral amino acids was observ-

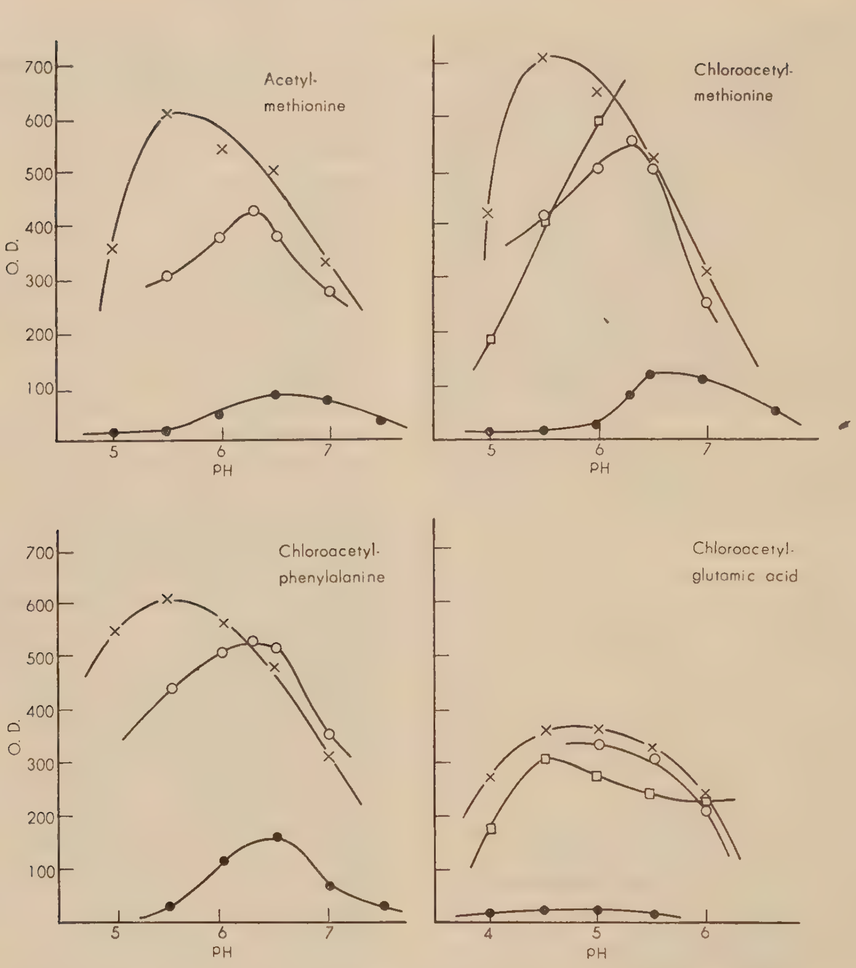

FIG. 2. pH-Activity Curves for Yeast Acylase Preparation in Individual Buffer Solutions.

The assay was made under standard conditions except for the buffer utilized. The symbols represent phosphate (○——○), Veronal (×——×), acetate (□——□), and citrate (●——●) buffers.

ed around pH 5.5.

The results indicate the need for specifying exactly the type of buffer used in the determination of yeast acylase activity.

Susceptibility of Acyl Amino Acids The susceptibility of the various acyl amino acids towards the yeast acylase preparation was studied in the initial rates of hydrolysis under standard conditions. The results obtained are shown in Table IV, in which the rates of hydrolysis were converted to relative susceptibility taking that of acetylmethionine as 100 for convenience of interpreting the results.

As in the case of mold acylase[6] previously studied, a number of acyl amino acids could be hydrolyzed by this yeast acylase preparation. Aliphatic acyl amino acids, such as acetylmethionine, chloroacetyl derivatives of alanine and leucine, were readily hydrolyzed. As for the acyl derivatives of aromatic amino acids, although chloroacetyl derivatives of phenylalanine and tyrosine were very suscep-

TABLE IV

RELATIVE SUSCEPTIBILITY OF ACYL AMINO ACIDS TO THE YEAST ACYLASE PREPARATION

Acyl amino acids	Susceptibility
Glyc-Glycine	23
Ac-Alanine	72
Clac- 〃	305
Ac-Leucine	21
Clac- 〃	227
Ac-Methionine	100*
Clac- 〃	274
Glyc- 〃	489
Ac-Glutamic acid	21
Clac- 〃	42
Ac-Aspartic acid	7
Clac- 〃	37
ε-Bz-α-Ac-Lysine	29
ε-Bz-α-Clac- 〃	52
Ac-Tryptophan	8
Clac- 〃	13
Ac-Phenylalanine	20
Clac- 〃	165
Clac-Tyrosine	208

The enzyme reaction was carried out under standard conditions. The prefixes represent acetyl (Ac), chloroacetyl (Clac), glycyl (Glyc), and benzoyl (Bz).

* 86 μM/hr/prot. N mg.

tible, acyl tryptophans were less susceptible to the preparation. Similarly to acylase preparations obtained from other sources such as mold and hog kidney, it was also observed that in this yeast preparation chloroacetyl derivatives were more rapidly hydrolyzed than acetyl derivatives.

Besides acyl amino acids, hydrolytic activity (a peptidase activity) of this preparation towards dipeptides such as glycylglycine and glycylmethionine was also studied. As a result, it was revealed that although glycylglycine was less susceptible, glycylmethionine was rapidly hydrolyzed.

Effect of Substrate Concentration The effect on the reaction rate of varying concentrations of acetylmethionine was examined under otherwise standard conditions. The same results have been plotted by the method of Lineweaver and Burk[8] for the determination of a Km value. As shown in Fig. 3, the substrate concentration giving half maximal velocity, i. e., Km was calculated to be around 8.3×10^{-3} M.

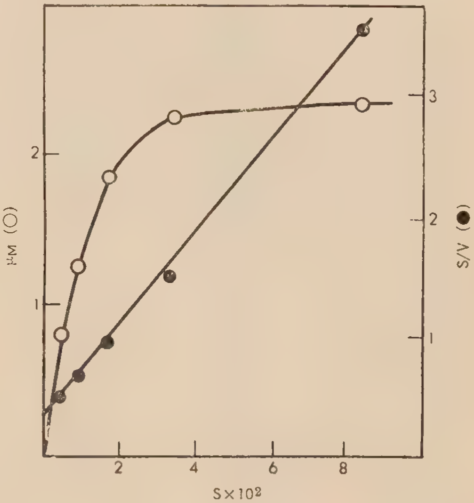

FIG. 3. Effect of the Concentration of Acetylmethionine to the Reaction Rate.

The reaction was similar to the standard condition except the varying substrate concentrations, and the incubation was carried out for 1.5 hours.

Effect of Metals and Inhibitors It had been observed that fungal[3,4] and bacterial acylase[7] preparations were activated by cobalt ion. Hence in order to study the effect of metals and inhibitors to the yeast acylase activity, acetyl and chloroacetyl methionines, and chloroacetylphenylanine were employed as substrates, and various divalent metal ions (metal chlorides), metal binders, and sulfhydryl reagents were added to the enzyme assay. Effect on the hydrolysis of glycylglycine and glycylmethionine was also investigated for comparison.

The results, showed in Table V, revealed that the effect towards glycylmethionine and all the acyl amino acids tested were almost identical except the effect on the hydrolysis of glycylglycine. Namely, although salts of

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[8] H. Lineweaver and D. Burk, *J. Am. Chem. Soc.*, **56**, 658 (1934).

TABLE V

EFFECT OF METALS AND INHIBITORS ON THE ENZYME ACTIVITY

Additions	Conc. (M)	Acetyl-methionine	Chloroacetyl-methionine	Chloroacetyl-phenylalanine	Glycyl-methionine	Glycyl-glycine
none		100	100	100	100	100
Cu	10^{-3}	7	5	2	23	17
Mg	10^{-2}	89	96	107		
	10^{-3}	99	90	103	93	97
	10^{-4}	98	89	103		
Ca	10^{-3}	96	83	96	23	40
Zn	10^{-2}	37	68	19		
	10^{-3}	71	75	72	68	45
	10^{-4}	85	98	82		
Mn	10^{-2}	12	11	4		
	10^{-3}	15	12	13	72	110
	10^{-4}	32	70	17		
Fe	10^{-3}	6	4	6	3	54
Co	10^{-2}	9	5	3		
	10^{-3}	85	65	81	48	680
	10^{-4}	92	92	102	98	
Ni	10^{-3}	22	8	14	52	125
NaF	10^{-3}	102	97	96	99	84
EDTA	10^{-3}	5	3	5	2	4
	10^{-4}	7				
	10^{-5}	15				
	10^{-6}	100				
NaCN	10^{-3}	110	102	101	104	86
PCMB	10^{-3}	6	6	5	28	63
Iodoacetate	10^{-3}	91	85	87	63	93

The assay was carried out under standard conditions modified by the addition. The activity of the respective substrate without addition was taken as control (100).

magnesium and calcium at the concentration tested, slightly affected the reaction, salts of copper, manganese, nickel, and iron were apparently inhibitory. Also, cobalt which was the activator for fungal and bacterial acylases and zinc were found slightly inhibitory. Despite this, any metal ion stimulating acylase activity of the yeast preparation could not be found under the conditions employed.

In the case of hydrolysis of glycylglycine, calcium was also proved inhibitory besides the salts of copper, zinc, and iron. Although cobalt, nickel and manganese ions were inhibitory to acylase activity, apparent stimulation was obtained with cobaltous ion, and salts of manganese and nickel also showed an enhancing effect though slightly on the hydrolysis of glycylglycine.

Marked inhibition by the metal chealating agent, ethylenediaminetetraacetic acid (EDTA) was observed in all the substrates tested. In the case of sulfhydryl reagents, although the inhibitory effect of iodoacetate appeared to a less extent, *p*-chloromercuribenzoate markedly inhibited the acylase activity. These results indicate that metal ion and sulfhydryl group play a certain role in the action of the yeast acylase.

For further study on the relation between the enzyme action and metal ion, the experiment was carried out with EDTA treated enzyme so as to investigate the reactivation by metal ions. The enzyme was treated with a 10^{-4} M concentration of EDTA at 37° for 30 minutes, which abolished almost all the activity. To this enzyme solution, 1.1×10^{-3} M concentration of metal chlorides were added and it was preincubated at 37° for 30 minutes. With this enzyme solution, activity was determined by employing acetylmethionine, glycyl-

TABLE VI

REACTIVATION OF THE INACTIVATED ENZYME BY METAL IONS.

Substrate	EDTA treatment	Activity in the Presence of Ions								
		None	Cu	Mg	Ca	Zn	Mn	Fe	Co	Ni
Acetyl-methionine	none	100	7	99	96	71	15	6	85	22
	treated	0	0	0	0	59	0	4	18	4
Glycyl-methionine	none	100	23	93	15	68	72	3	48	52
	treated	0	4	0	0	18	28	0	16	13
Glycyl-glycine	none	100	17	97	6	45	110	54	680	125
	treated	10	22	12	4	24	50	40	137	36

The enzyme assay was carried out under standard conditions except for the preincubation of EDTA treated enzyme solution with metal ions. The activity of the respective substrate without EDTA treatment and none addition of metal ions was taken as control 100.

methionine and glycylglycine as the substrates. Activity was represented as the percentage of the control without EDTA treatment for comparison.

As shown in Table VI, although only partial reactivation was recognized, complete reactivation as observed in the case of mold acylase,[3] was not attained under such conditions employed.

Effect of Temperature For the purpose of studying on the effect of temperature to the enzyme reaction, the extent of hydrolysis of acetyl-, chloroacetylmethionines and chloroacetylphenylalanine was determined at respective optimal pH

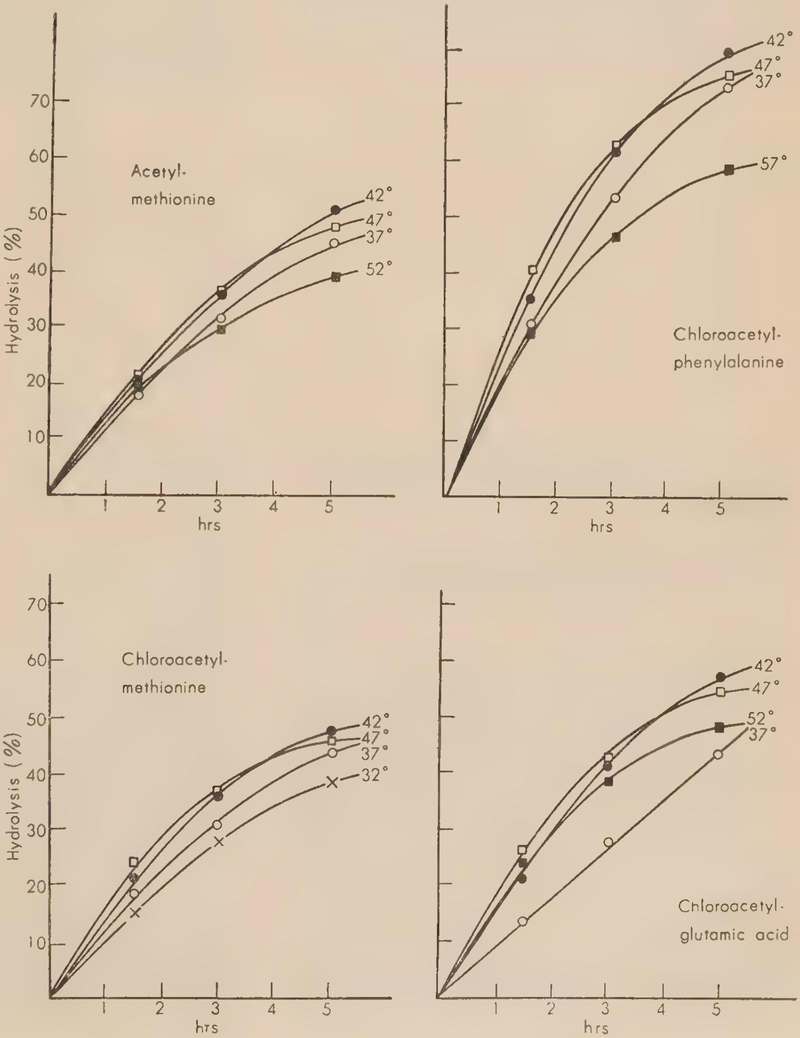

FIG. 4. Effect of Time and Temperature on the Hydrolysis by the Enzyme Preparation from Brewer's Yeast.

The enzyme assay was carried out under standard conditions modified by varying incubation terms and temperature.

and varied temperature instead of at 37° as in standard conditions. The results, indicated in Fig. 4, disclosed that maximal rate of hydrolysis was observed at 47° within 3 hours in all of the substrates tested.

Optical Specificity Optical specificity of this acylase preparation from yeast was investigated, since some of the bacterial enzymes had been known to act on D-amino acid derivatives. The enzymatic reaction employing 0.025 M chloroacetyl-L-methionine and 0.05 M chloroacetyl-DL-methionine, as the substrate was simultaneously carried out under standard conditions, and the extent of hydrolysis was determined. As indicated in Fig. 5, in either case, the reaction stopped at the cleavage of 25 mM of the substrate, that is, 100% hydrolysis of the L-form substrate and 50% hydrolysis of the racemic substrate, in which no further hydrolysis was occurred by prolonged incubation. This result indicates that the yeast preparation is optically specific and acts only to the L-form of acyl amino acid and not on the D-isomer. While, on the other hand it appears that the presence of D-isomer does not appriciably affect the rate of hydrolysis of the acyl-L-amino acid within the sensitivity of the method employed.

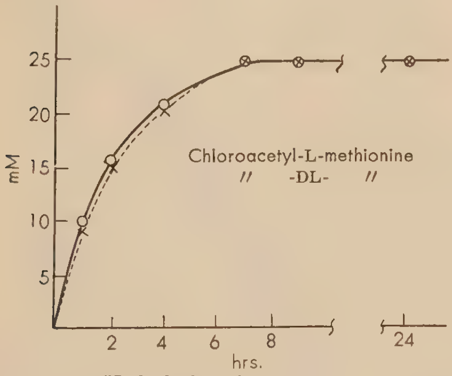

FIG. 5. Hydrolysis of Chloroacetyl-L- and Chloroacetyl-DL-methionines by the Yeast Acylase Preparation.

The enzymatic reaction employing 0.025 M chloroacetyl-L-methionine and 0.05M chloroacetyl-DL-methionine carried out at pH 6.0 under standard conditions. Rate of hydrolysis is expressed by the liberated amount of amino acid.

DISCUSSION

The authors' studies performed on the characteristics of the yeast acylase preparation obtained from brewer's yeast reveal that the preparation resembles other acylase preparations such as fungal and bacterial acylases in several features. The yeast preparation is able to hydrolyze acyl derivatives of various types of aliphatic and aromatic amino acids such as mold acylase, previously studied by the authors. Namely, a callyl amino acids tested were hydrolyzed more or less with the preparation, and chloroacetyl derivatives were more susceptible than the acetyl derivatives. Futhermore optical specificity is also similar to other acylases previously studied, the L-form of acyl amino acids is selectively hydrolyzed. These results indicate that the yeast acylase preparation may be used for the purpose of enzymatic resolution of racemic amino acids.

The inhibitions by EDTA and PCMB indicates that a metal ion and sulfhydryl group play a role in this enzyme action. Although the acylase preparations from other sources were activated by cobaltous ion, any appreciable stimulation was not observed by the addition of divalent metal ions including cobalt in the yeast preparation under the conditions employed. Also, the enzyme previously treated with EDTA was not appreciably reactivated with divalent metal ions. These facts may be apprehended that the enzyme is irreversibly denaturated by EDTA treatment or the enzyme requires other metal ions than the divalent metal ions tested.

Besides acyl amino acids, the yeast preparation employed can hydrolyzed glycylmethionine readily and glycylglycine in a slow rate. In this study, no attempt was made to separate the acylase activity from dipeptidase activity. Although, the influence of metal ions towards acyl amino acids and glycylmethionine appeared to be almost the same, the effect to hydrolysis of glycylglycine differed. For instance, the latter was activated with cobalt ion but the others were rather inhibited with the ion. However, it can not be decided from the above fact whether this peptidase action especially to glycylglycine, is due to a different enzyme than acylase at the present state of investigation. It is also possible that the yeast preparation contains several enzymes which are capable of hydrolyzing acyl amino acids, so the decision as to whether or not the acylase activity in this enzyme preparation is due to the occurrence of single or more specific acylases has to be held until the enzyme is obtained in a further purified state.

Through the present studies, the occurrence in yeast of acylase activity has been confirmed for the first time, and disclosing some enzymatic properties. The authors intend to continue the studies in order to clarify the biological significance of acylase activity occurring in various organisms.

Acknowledgements The authors are grateful to Prof. H. Mitsuda of Kyoto University, Dr. M. Fujisawa of this Laboratory, and Dr. S. Yamada of Tokyo Research Laboratory for their helpful advice and encouragement. They are also indebted to Dr. Y. Kuroiwa, Amagasaki Laboratory, Kirin Brewing Co. for generously supplying the yeast used in this work.

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Studies on Phenolic Lactones

Part I. Synthesis of α-Piperonylidene-β-piperonyl-butyrolactone

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Condensation of piperonal with diethyl piperonylsuccinate afforded α-(3,4-methylenedioxyphenyl-hydroxymethyl)-β-(3, 4-methylenedioxybenzyl)-succinic acid, m.p. 173～5°C. Dehydration with *p*-toluenesulfonic acid or acetic anhydride gave two kinds of α-piperonylidene-β-piperonyl succinic anhydrides, m.p. 167–8°C and 115–7°C, respectively. Reduction of α-piperonylidene-β-piperonyl-succinic acid thioethyl methyl ester by Raney Ni catalyst followed by saponification and lactonization afforded α,β-dipiperonyl butyrolactone, m.p. 107～8°C, and a small amount of α-piperonylidene-β-piperonyl-butyrolactone, m.p. 154～5.5°C.

Hibalactone is the neutral component isolated from dried leaves of *Chamaecyparis obtusa* Sieb et Zucc by Masumura.1) Catalytic hydrogenation of this substance affords isohinokinin,2) which is converted into hinokinin by base catalyzed isomerization. The ultraviolet2)

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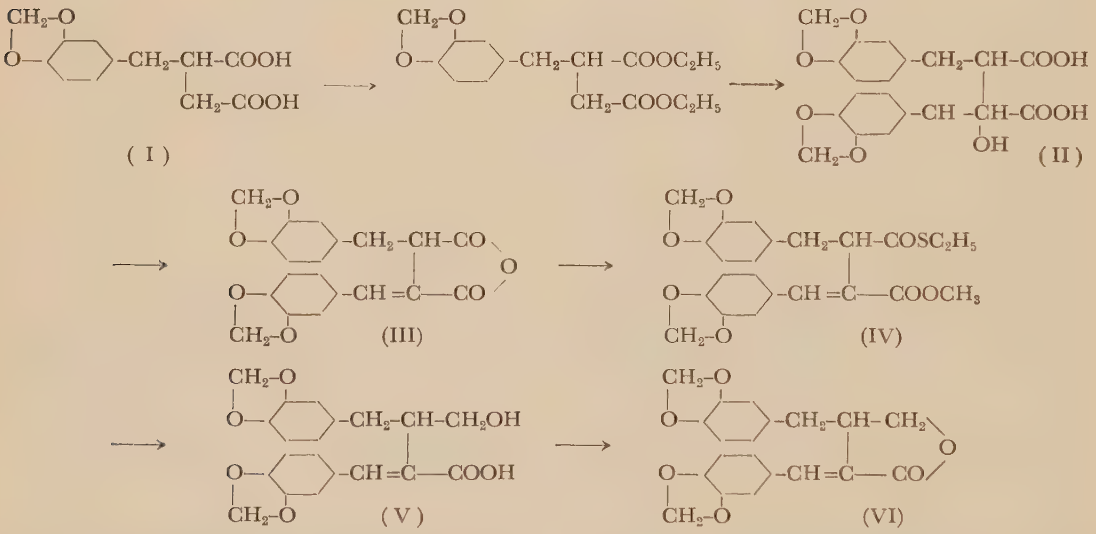
1) M. Masumura, *Bull. Chem. Soc. Japan*, **76**, 423 (1955).

2) M. Masumura, *ibid*, **77**, 635 (1955).

and infrared[3] absorption spectra show that hibalactone must possess the α,β-unsaturated-γ-lactone formulations. Based upon these results and other examinations the structure of hibalactone was concluded to be α-(3,4-methylenedioxybenzylidene)-β-(3, 4-methylenedioxybenzyl)-butyrolactone. It may also be stated that hibalactone is identical with savinine obtained from *Juniperus savina* by Hartwell et al.[4~7] Because of its synergistic activities[8] we attempted to synthesize it by applying various methods.

Hinokinin, the hydrogenated product of hibalactone, was synthesized by the reduction of α,β-di-(3,4-methylenedioxybenzyl)-succinic anhydride with amalgumated aluminum.[9] But as reduction with amalgumated aluminum generally hydrogenates the double bond conjugated to the carbonyl group, α-(3,4-methylenedioxybenzylidene)-β-(3, 4-methylenedioxybenzyl)-succinic anhydride was assumed to be reduced to hinokinin by amalgumated aluminum.

The conversion of carboxylic acids into primary alcohols is successfully performed by the reduction of thioalkyl ester with Raney Ni,[10] and Masamura synthesized α,β-dibenzylbutyrolactone by this method.[11] However as Raney Ni catalyst generally hydrogenates the double bond, the presence of -S- in thioethyl ester would be expected to inhibit the hydrogenation of the double bond; for this purpose the authors therefore attempted the Raney Ni reduction of thioethyl ester as:



As it will be described in the experimental part, most of the double bond was hydrogenated and a large part of reduction product was hinokinin, the yield of α-piperonylidene-β-piperonyl-butyrolactone being poor in contrary to the authors' expectations.

Stobbe condensation of piperonal with diethyl piperonylsuccinate afforded hydroxy acid (II), the structure of which was confirmed

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3) M. Masumura, *ibid*, **76**, 425 (1955).

4) J. L. Hartwell, J. M. Johnson and D. B. Fitzgerald, *J. Am. Chem. Soc.*, **75**, 235 (1953).

5) A. W. Schrecker and H. L. Hartwell, *ibid*, **76**, 4896 (1954).

6) T. Kariyone and K. Isoi, *J. Pharm. Soc, Japan*, **74**, 1312 (1954).

7) M. Masumura and F. S. Okumura, *J. Am. Chem. Soc.*, **77**, 1906 (1955).

8) H. Matsubara, This Bulletin, **21**, 132 (1955); H. Matsubara, *Bull. Institute of Insect Control*, **22**, 345 (1957).

9) R. D. Howorth et al., *J. Chem. Soc.*, 1985 (1938); S. Keimatsu et al., *J. Pham. Soc. Japan*, **55**, 775 (1935).

10) O. Jeger, J. Norymberski, S. Szpilfogel, V. Prelog, *Helv. Chim. Acta* **29**, 684 (1956).

11) M. Masumura, *J. Chem. Soc. Japan*, **76**, 1381 (1955).

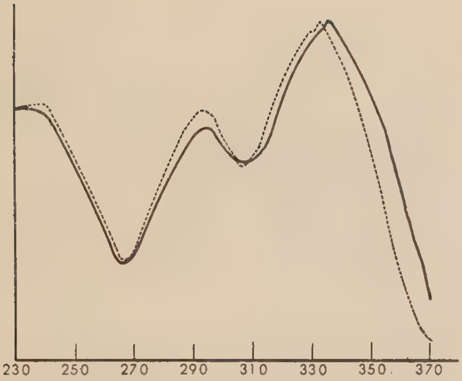


FIG. 1. Ultraviolet Absorption Spectra of Hibalactone and Isohibalactone.

- - - Hibalactone (authentic)
—— Isohibalactone (synthetic) (chloroform solution)

from the elementary analysis and infra red spectrum. Dehydration of the acid with *p*-toluenesulfonic acid afforded unsaturated acid anhydride (III), m.p. 167°C. The acid anhydride obtained from hydroxy acid (II) by boiling with acetic anhydride was also unsaturated anhydride but its melting point was 117°C. This difference was explainable from the cis-trans isomerisms.

The reaction of acid anhydrides with sodium mercaptide followed by methylation was assumed to afford either (IV) or (VII). Reduction of (VII) with Raney nickel catalyst will lead to β,γ-unsaturated-γ-lactone (VIII), while (IV) will lead to α,β-unsaturated-γ-lactone

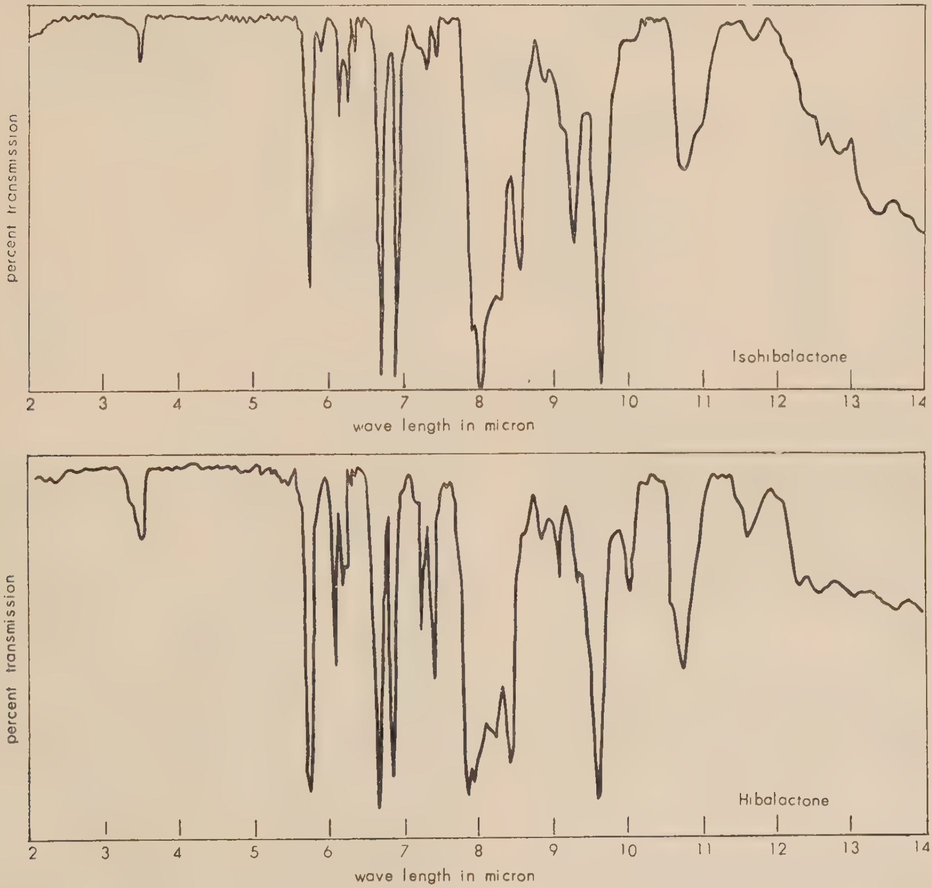


FIG. 2. Infrared Absorption Spectra of Authentic Hibalactone and Synthetic Isohibalactone. (Chloroform solution).

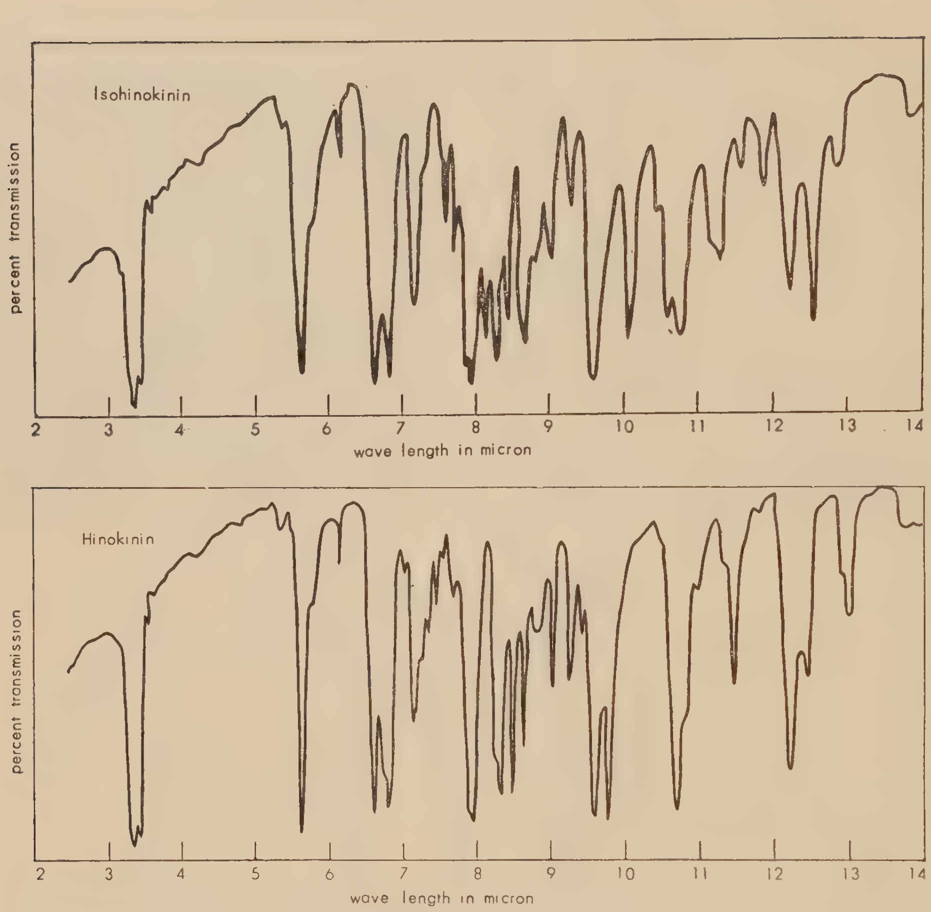
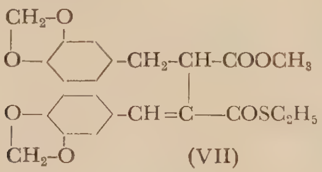
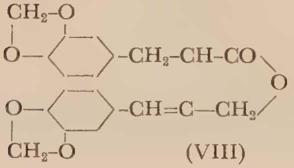


FIG. 3. Infrared Absorption Spectra of Isohinokinin and Hinokinin (Nujol mull).

(VI). Starting from the acid anhydride of m.p. 117°C, the authors obtained hinokinin and an unsaturated lactone, while from the other acid anhydride, m.p. 167°C, only hinokinin was obtained.



(VII)



(VIII)

The infra red spectrum of the synthesized unsaturated lactone showed a pronounced stretching vibration at 6.11 μ which is characteristic of double bond conjugated to carbonyl group. The shift of the carbonyl band toward lower frequencies from 5.66 μ to 5.73 μ is also characteristic of α,β-unsaturated-γ-lactone. The ultraviolet spectrum showed strong absorption at 336 mμ which showed the conjugation of carbonyl group, double bond and benzene nucleus. From these results and elementary analysis the structure of synthesized lactone was confirmed to be (VI) which corresponded to hibalactone or savinin, and the structure (VIII) could be excluded. Consequently, at least some part of staring thioethyl-methylester was confirmed to have structure (IV). But the infra red spectrum of sythesized unsaturated lactone did not agree completely with an authentic specimen. From these results the synthesized α-piperonylidene-β-piperonyl-butyrolactone was assumed to be the geometric isomer of racemic hibalactone and the authors designated it as isohibalactone.

The authors wish to express their thanks to Dr. Masumura, Tokushima University, for kindly supplying us the authentic hibalactone. Indebts are also due to the Laboratory of Microanalysis, Department of Agricultural Chemistry, University of Tokyo, for the microanalyses.

EXPERIMENTAL

All melting points were uncorrected.

Piperonyl-succinic Acid

a) via piperonylchloride. In a 500-ml three-necked flask was placed 200-ml of dried ether and 2.8 g (0.074 mole) of lithium aluminum hydride. A solution of 40 g (0.267 mole) of piperonal in 100 ml of dry ether was added from the dropping funnel at a rate so as to produce gentle reflux. Agitation was continued for 30 minutes after all the aldehyde had been introduced, and the excess hydride was then destroyed by careful addition of ethylacetate and water. With constant stirring 100 ml of 10% hydrochloric acid was then slowly added. The ether layer was separated, and the solvent was removed by distillation under atmospheric pressure. Forty grams of crude piperonyl alcohol was obtained as a residual oil which gradually solidified. The crude alcohol was directly chlorinated as follows.

Crude alcohol (40 g) was dissolved in 200 ml of benzene and 20 g of anhydrous sodium sulfate was added. While the mixture was being cooled at 0–5°C, dry hydrogen chloride was passed for 30 minutes and left standing for several hours in the cold. The filtered solution was washed well with water. Evaporation of the solvent left 42 g of crude piperonyl chloride which gradually solidified, m.p. 20°C. After recrystallization from benzene-petroleum ether, the yield was 36 g (79% from piperonal), m. p. 22–3°C.

Metalic sodium (4.5 g) was dissolved in 100 ml of absolute ethylalcohol and 48 g of diethyl carbethoxysuccinate was added. Then 36 g of piperonyl-chloride in 50 ml of ethyl alcohol was added at such a rate that a gentle reflux was maintained. The mixture was heated under reflux for 2 hours. A solution of 40 g of potassium hydroxide in 100ml of ethyl alcohol and 20ml of water was added dropwise and refluxed for an hour. One hundred milliliters of water was added and the alcohol was distilled off. After the removal of neutral substances by ether extraction, the aqueous solution was acidified with hydrochloric acid and was extracted thrice with ether. The combined extract was washed with water and the ether distilled off. The residual semisolid was decarboxylated by heating to 170–180°C for 30 minutes. As the acid anhydride was formed in this treatment, the decarboxylated product was hydrolyzed by boiling with 24 g of potassium hydroxide and 100 ml of water for an hour. Acidification with hydrochloric acid and ether extraction afforded 36 g (68 %) of piperonyl-succinic acid, m.p. 105–110°C. Recrystallization from ethylacetate-petroleum benzine gave 30 g of pure product, m.p. 135–6°C.

b) via piperonylidenesuccinic acid. Condensation of 54 g of piperonal with 63 g of diethyl succinate in 300ml of absolute methylalcohol on the presence of 21 g of sodium by the method of Campbell et al.[12] gave 70.5 g of crude piperonylidenesuccinic acid, m. p. 180°C. Crude acid was hydrogenated at room temperature and 90 atm. in slightly alkaline aqueous solution over 20 g of Raney nickel catalyst and the filtered solution was acidified with hydrochloric acid. The precipitated crude acid, m. p. 115°C, which was recrystallized from hot water, weighed, 51g, m.p. 134–6°C.

α-(3, 4-Methylenedioxyphenyl-hydroxymethyl)-β-(3,4-methylenedioxybenzyl)-succinic Acid. Piperonylsuccinic acid (39 g) was esterified by refluxing with 100 ml of ethylalcohol, 200 ml of benzene and 20 ml of sulfuric acid for 4 hours. The upper-layer was separate, washed with water, aqueous sodium bicarbonated solution and water, and the solvent was removed in vacuo. Crude ester (40 g) was obtained as brownish oil.

To a cooled solution of 4.8 g of sodium in 100 ml of anhydrous ethyl-alcohol was added a mixture of 25.5 g of crude diethyl piperonylsuccinate and 12.4 g of piperonal and the mixture was allowed to stand over night. After refluxing for two hours, 200 ml of water was added and alcohol was removed by distillation. Neutral substances was removed by the ether extraction, and the aqueous solution was acidified with hydrochloric acid. Ether extraction and evaporation of ether left 31.5 g of oily residue, upon which treatment with 50 ml of ethylalcohol gradually separated 16.5 g of pale yellow needles, m.p. 170–2°C. Recrystallization from methylalcohol afforded colorless prisms, m. p. 173–5°C. From the analytical value and infrared spectrum this acid was assumed to be hydroxy acid and not unsaturated acid. *Anal.* Found: C, 60.62; H, 4.69, Calcd. for $C_{20}H_{18}O_9$ requires C, 59.8; H, 4.5 %.

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[12] K. N. Campbell, J. A. Cella and B. K. Campbell, *J. Am. Chem. Soc.*, **75**, 4681 (1953).

α-Piperonylidene-β-piperonylsuccinic Anhydride

a) α-(3, 4-methylenedioxyphenyl-hydroxymethyl)-β-(3,4-methylenedioxybenzyl)-succinic acid (1.0 g) was dissolved in 60 ml of xylene and small amount of *p*-toluenesulfonic acid was added. After refluxing for an hour the cooled solution was washed with water and concentrated to dryness in vacuo. The yellow crystalline residue was recrystallized from benzene-petroleum benzine, m.p. 167–8°C, yield 0.85 g. *Anal.* Found: C, 65.79; H, 4.22, Calcd. for $C_{20}H_{14}O_7$ requires: C, 65.6; H, 3.8 %.

The infra-red spectrum showed the presence of a double bond conjugated to the carbonyl group.

b) A mixture of 10 g of α-(3, 4-methylenedioxyphenyl-hydroxymethyl)-β-(3, 4-methylenedioxybenzyl)-succinic acid and 30 ml of acetic anhydride was boiled for 4 hours. The yellow solution was concentrated to syrup in vacuo. The residual mass gradually solidified, m.p. 113°C. Recrystallization from benzene-petroleum benzine afforded 9.0 g of yellow anhydride, m.p. 115–7°C. *Anal.* Found: C, 65.79; H, 3.95; Calcd. for $C_{20}H_{14}O_7$ requires C, 65.6; H, 3.8 %.

α-Piperonylidene-β-piperonyl-succinic acid β-thioethyl-α-methyl ester. To a suspension of 1.15 g of sodium sand in 50 ml of dry benzene was added 9 g of ethyl mercaptan and the mixture was agitated for several hours untill all the sodium was converted to ethyl mercaptide. Acid anhydride of m.p. 117°C (8.0 g) in 50 ml of benzene was added to the above suspension and stirred 3 hours at room temperature and left aside overnight. The reaction mixture was extracted thrice with each 30 ml portions of 10% aqueous sodium carbonate solution. The alkaline extract was acidified with dil. sulfuric acid and extracted with benzene. Combined extract was washed with water and the solvent was distilled off. Monothioethylester obtained as viscous oil; weighed 8.1 g.

Crude monothioethyl ester (8.1 g) was dissolved in 100 ml of ether and esterified with ethereal solution of diazomethane prepared from 7 g of nitrosomethylurea. Evaporation of the solvent left 8.1 g of methyl-thioethylester as syrup. The crude product was directly used in the following reaction.

α-Piperonylidene-β-piperonyl-butyrolactone (Isohibalactone). In a 500 ml three necked flask were placed 200 ml of 90% ethylalcohol and 30 g of Raney Ni catalyst. A solution of 8.1 g of methyl-thioethyl ester in 100 ml of 90% ethyl alcohol was added and agitation was continued for 3 hours at room temperature and afterwards for one hour at 80°C. The filtered solution concentrated to syrup in vacuo, weighed 6.0 g. The residual oil was boiled with 150 ml of 5% aqueous sodium hydroxide solution for 2 hours. After cooling the neutral substances were removed by ether extraction and the mother liquor was acidified with 18 ml of conc. hydrochloric acid and warmed on a water bath for 30 minutes to complete lactonization. The mixture was poured into excess of aqueous sodium bicarbonate solution and extracted four times with chloroform. The combined extracted was washed with aqueous sodium bicarbonate solution, then with water and dried over anhydrous sodium sulfate. Evaporation of the solvent left 3.2 g of oily product. This was dissolved in 5 ml of chloroform and 10 ml of hot ethyl alcohol was added. After cooling in a refrigerator overnight the separated crystalline needles were collected by filtration and washed with ethyl-alcohol, yielded 1.0 g, m.p. 145–7°C. Recrystallization from ethylacetate-ethylalcohol afforded colorless needles, m.p. 154–155.5°C. *Anal.* Found: C, 67.92; H, 4.55, Calcd. for $C_{20}H_{14}O_6$ (hibalactone) requires: C, 68.1; H, 4.5 %.

The ultraviolet absorption spectrum showed the maximum at 293, 334 mμ, minimum at 265, 305 mμ (authentic specimen showed max. at 292, 334 mμ, min. at 265, 305 mμ). The infrared spectrum showed the presence of α, β-unsaturated-γ-lactone (5.76, 6.11 μ). From these results the product was assumed to be α-piperonylidene-β-piperonyl-butyrolactone.

***dl*-Hinokinin**

a) α-Piperonylidene-β-piperonyl-butyrolactone was hydrogenated at room temperature and atmospheric pressure over 10% Pd-C in acetic acid. The filtered solution was concentrated to syrup and the residual mass was recrystallized from ethylalcohol. White needles, m.p. 115–6°C, was obtained. *Anal.* Found: C, 67.42; H, 5.11, Calcd. for $C_{20}H_{18}O_6$ requires: C, 67.8; H, 5.1 %. This lactone was assumed to be *dl*-isohinokinin.

Saponification of this lactone with methanolic potash followed by lactonization isomerized isohinokinin to hinokinin and consequently *dl*-hinokinin, m. p. 107–8°C, was obtained. (m. p. 108°C as reported by Howorth) *Anal.* Found: C, 67.36; H, 5.30, Calcd. for $C_{20}H_{18}O_6$ requires; C, 67.8; H, 5.1%. The mixed melting point with an authentic specimen was also 107–8°C.

b) The filtrate from the α-piperonylidene-β-piperonyl-butyrolactone was concentrated to a small volume and left to standing several days in a refrigerator. The whole mass was gradually solidified, m.p. 95–6°. Recrystallization from ethylalcohol gave colorless prisms, m.p. 107–8°C. The mixed melting point of it with an authentic specimen was also 107–8°C.

[Bull. Agr. Chem. Soc. Japan, Vol. 22, No. 4, p. 233~238, 1958]

Genetic Background of Kojic Acid Production Studied with Metabolic Mutants of *Aspergillus oryzae*

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Received March 29, 1958

In order to elucidate the genetic background of sugar metabolism by *Aspergillus oryzae*, three kinds of metabolic mutants were prepared by induced mutation. Though these mutant strains generally produced a few ferric chloride-positive substance, it is not yet known whether the substance is a precursor of kojic acid or not. The heterocaryons between a kojic acid producer and a hetero-fermentative-type strain produced kojic acid in the same amount as the parental producer, while the heterocaryons between a kojic acid producer and a respiratory-type strain produced a less amount of kojic acid. Selection of fitter-type nuclei during the course of propagation is considered to be a possible reason.

As it is well known, *Aspergillus oryzae-flavus* group of fungi metabolize sugars through the three alternative pathways, respiration, alcoholic fermentation, and oxidative fermentation. For example, the strain studied here, *Asp. oryzae* 51, accumulates ethylalcohol and kojic acid as its main products when cultivated in malt extract medium by stationary culture.

In this paper, the author intends to study the genetic background of three alternative pathways by genetics means. The systems were separated into each other by induced mutation, and then combined again to produce heterocaryons. The advantages as well as the disadvantages of the application of these techniques for the study of fungus physiology are discussed.

METHODS AND TERMINOLOGY

Strains: Two biochemical mutants of *Asp. oryzae* 51, a lysineless mutant No. 22 (*Lys*–22) and a leucineless mutant No. 23 (*Leu*–23), were employed as the starting materials. Strain *Lys*–22 produced kojic acid in an amount of from 2,000 to 5,000 γ/ml and strain *Leu*–23 from 200 to 1,000 γ/ml. From the results of paper chromatographic examination, no significant accumlation of organic acid except that of kojic acid was observed. The metabolic mutants derived from these original strains will be described later.

Media: Cultivation was made in malt extract medium (Bllg 8°) in 300 ml Erlenmeyer flasks (100 ml-medium) or test tubes (10 ml-medium). In the case where test tubes were employed for the purpose, the tubes were inclined so as to produce a larger surface of liquid. Excepting a special case which is to be mentioned later, one crop of conidia was inoculated from each slant culture.

Measurement of fermentation products: Kojic acid was measured by the method reported elsewhere.1) Ethylalcohol produced and sugar consumed were determined by the permanganate method and the Bertrand method, respectively. Acidity was expressed as ml of 1/10 N NaOH per 10 ml sample.

Terminology of metabolic mutants: The original strains and their mutants were cultivated under both aerobic and anaerobic conditions. Aerobic culture implies ordinary stationary culture, while, in anaerobic culture, the flasks were shaken by hand several times each day so that the mycelia might be always in the liquid.

The strains growing on the surface of liquid medium and producing a large amount of kojic acid and a low quantity of ethylalcohol under aerobic conditions are termed as wild-type or kojic acid producer-type regardless of the growth factor requirement.

The strains growing on the surface of medium and forming no kojic acid under aerobic conditions are termed as respiratory-type or kojic acid non-producer-type.

Meanwhile, the strains growing only in liquid even under aerobic conditions and producing ethylalcohol as the main product are termed as homo-fermentative-type or respiratory deficient-type. These strains are in principle kojic acid non-producer. One strains (*Leu*-1) did not produce any kojic acid but instead accumulated ethylalcohol in a comparatively large amount. This strain is especially termed as hetero-fermentative-type.

All the strains tested produced ethylalcohol in a yield of 30–35 per cent of the consumed sugar. Therefore, it is evident that the glycolytic system of these metabolic mutants is in a state as healthy as that of the wild-type strains.

RESULTS

1. Induction of metabolic mutants.

Kojic acid non-producers were frequently obtained (one mutant among 50 survivors) from strains *Leu*-23 by treatment with ultraviolet light. One of the mutants thus obtained, *Leu*-31, produced kojic acid in a negligible amount (200 γ/ml as the maximum) when cultivated in the four media reported suitable for the acid production (Sakaguchi's2), Katagiri and Kitahara's3), malt extract, and Koji

TABLE I. FERMENTATION PRODUCTS FROM MALT EXTRACT (Bllg. 8°)

1. Aerobic Culture

Strains	*Lys*-22 wild	*Lys*-25 resp.	*Lys*-2 homoferm.	*Leu*-31 resp.	*Leu*-1 heteroferm.
pH	3.6	3.6	4.6	3.6	3.8
Growth	surface	surface	in liquid	surface	surface $(\frac{1}{3})$
Acidity	4.60	2.78	2.54	3.80	4.02
Alcohol (g/100 ml)	0.88	0.29	1.38	0.52	1.35
Kojic acid (〃)	0.19	0.01	0.01	0.01	0.01
Mycerial weight (〃)	0.84	1.00	0.24	0.79	0.52
Sugar consumed (〃)	4.85	4.05	3.75	3.05	4.75

2. Unaerobic Culture

Strains	*Lys*-22 wild	*Lys*-25 resp.	*Lys*-2 homoferm.	*Leu*-31 resp.	*Leu*-1 heteroferm.
pH	4.2	4.4	4.4	4.2–4.4	4.2
Growth	in liquid	in liquid	in liquid	in liquid	in liquid
Acidity	3.60	3.96	3.04	3.04	3.62
Alcohol (g/100 ml)	1.08	1.29	1.35	0.88	0.88
Kojic acid (〃)	0.01	0.01	0.01	0.01	0.01
Mycerial weight (〃)	0.26	0.28	0.24	0.33	0.26
Sugar consumed (〃)	2.85	3.75	3.50	3.00	0.70

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1) Y. Ikeda, K. Nakamura, K. Uchida, and C. Ishitani, *J. Gen. Appl. Microbiol.*, **3**, 93 (1957).

2) K. Sakaguchi, *J. Agr. Chem. Soc. Japan*, **8**, 265 (1932).

3) H. Katagiri and K. Kitahara, Memories of Coll. Agr. Kyoto, No. 26 (1933).

extract media). Strains *Leu*–1 was also a non-producer, but in contrast to strain *Leu*–31 it produced alcohol in a considerably large amount. As the strain formed mycelial mat on the surface of a liquid medium to an extent of one-third of that of the wild-type strains, it is thus designated as the hetero-fermentative-type in accordance to the criteria previously given.

In the same way, strain *Lys*–22 was treated with ultraviolet light or acriflavin. Among the 470 survivors after treatment with acriflavin (10 mg/ml in final concentration, for 5 hours, surviving rate about 1 %), only one kojic acid non-producer, *Lys*–25, was obtained. Although any trials attempted to obtain a respiratory deficient mutant by the treatment resulted in failure, the author happened to find out a spontaneous mutant (*Lys*-2) in one of the plate cultures. The mutant was aconidial in phenotype and required L-lysine for growth. When cultivated in a liquid medium, it growed in liquid without forming any mycelial mat on the surface. The amount of alcohol produced in aerobic conditions was almost equal as that in unaerobic conditions. For these reasons, the strain was considered to be homo-fermentative-type.

Table I shows the results of comparative studies carried out with wild-type and mutant type strains.

2. Metabolic products from the mutants

The fermented broth prepared in parallel with those mentioned above were analysed by the paper chromatographic techniques, using a butanol-acetic acid-water mixture (4 : 1 : 5) as the solvent and 0.2 per cent brom-cresol-green solution or 5 per cent ferric chloride solution as the development agent. As a matter of fact, strains *Lys*–22 (wild-type), *Leu*–31 (respiratory-type), and *Lys*-25 (respiratory-type) did give rise to two spots corresponding to kojic acid and succinic acid. However, because the amount of kojic acid produced by strains *Leu*–31 and *Lys*–25 was quite low (less than 100 γ/ml), and the leakage of genetic block is not unusual in microorganisms,[4] the production was considered to be due to the incomplete block of the metabolic pathway.

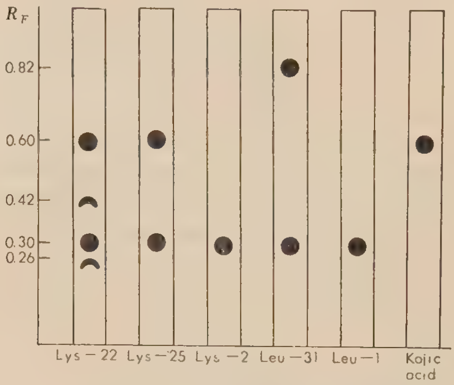

FIG. 1. Paper Chromatographic Examination of Ferric Chloride Positive Substances.

Next, as the author's interest was directed to the possible precursor substance(s) of kojic acid which might have been accumulated by the genetic block, the mycelia were first homogenized and then extracted with hot acetone. The syrup after evaporation was analysed by a paper chromatogram. As a result, four ferric chloride positive substances were detected on the paper in the case of strain *Lys*–22, two substances in *Leu*–31, one substance in *Leu*–1, *Lys*–2, and *Lys*–25. In Figure 1, the substance at R_F value 0.60 is kojic acid, while those at 0.26, 0.30, 0.42, and 0.82 are unidentified. It is of interest that the substance giving R_F value 0.30 was found in common among the five strains; however it is still unknown whether the substance is a precursor of kojic acid or not.

3. Evidences that the variations are actually induced by mutational events

Although the strains induced by ultraviolet light or acriflavin treatment have so far been referred to as mutants, evidences supporting the view will be described here.

Heterocaryons were produced by the modi-

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[4] D. M. Bonner, *Cold Spr. Harb. Symp. Quan. Biol.*, **16**, 143 (1951).

TABLE II. DISTRIBUTION OF HETEROCARYON FORMING ABILITIES AMONG METABORLIC MUTANTS

	Lys–22	*Lys*–25	*Lys*–1
Leu–23	+ (0.2%)	+ (0.01%)	+* (0.01%)
Leu–31	+ (0.01%)	— (<0.01%)	— (<0.01%)
Leu– 1	+ (0.1-0.2%)	— (<0.01%)	— (<0.01%)

* Evidenced only recently.

fied Sermonti method, employing one per cent koji extract agar as a limitedly supplemented medium. Table II shows the frequencies of hetero-caryon formation thus obtained. The respiratory-type or fermentative-type strains were less incompatible than the wild strains, and some of them were completely incompatible. Upon conidiospore formation, the heterocaryons yielded three kinds of progenies, both component types and their heterocaryon-type. As a rule, the morphological and biochemical characters of the component-type segregants were the same as those used for the heterocaryon formation. It is clear, therefore, that the variation induced by the treatments are due to genetic changes occuring at the nuclear genes.

As exceptions, among the 407 leucineless segregants from the heterocaryon between strains *Lys*–22 and *Leu*–1, five strains produced kojic acid in an amount of 1,000 γ/ml at the first cultivation after segregation. However, the production converted to the original state (less than 100 γ/ml) after two sequent transfers. A similar phenomenon was found in conidial color. Among the 214 lysineless segregants from the heterocaryons between *Lys*–22 (olive yellow)[5] and *Leu*–31 (dark olive buff), 11 strains were partially equipped with *Leu*–31 characters. Since these intermediate characters disappeared soon after one transfer, this temporary variation as well as the modification found in kojic acid production may be due to the non-autonomous segregation reported by Pontecorvo.[6]

The second support that the variation from kojic acid producer to a non-producer is a real genetic one was produced by the genetic recombination test, but as details have been reported elsewhere[7] there remains nothing to be added. Furthermore, the submerged-type respiratory deficient strain (*Lys*–2) is not a cytoplasmic mutant as it has been evidenced by the heterocaryon test.

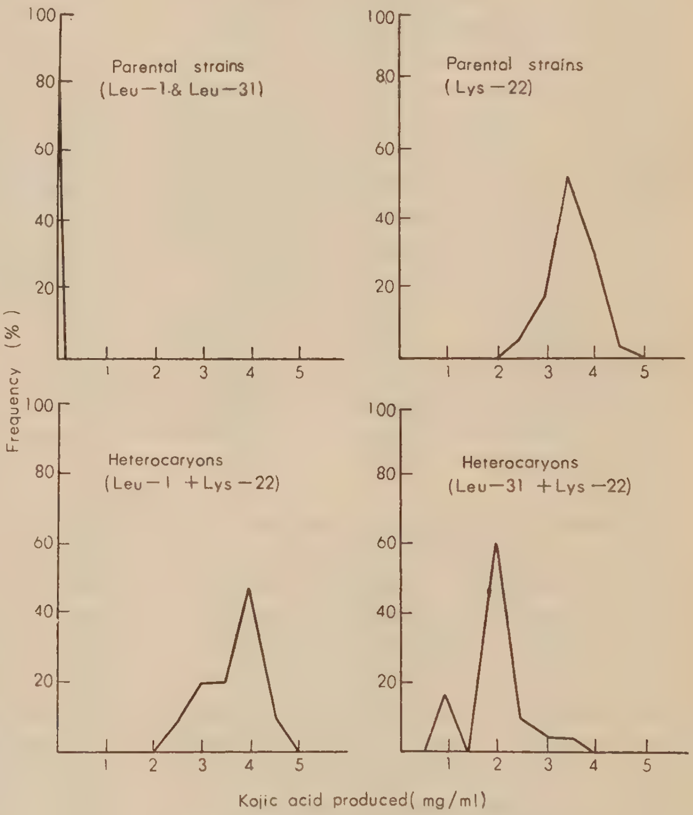

Fig. 2. Production of Kojic Acid by Strains *Lys*–22, *Leu*–31, *Leu*–1, and their heterocaryons.

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[5] R. Ridgway, Color Standard and Color Nomenclature, (1912).

[6] G. Pontecorvo, *Cold Spr. Harb. Symp. Quan. Biol.*, **11**, 193 (1946).

[7] Y. Ikeda, C. Ishitani, and K. Nakamura, *J. Gen. Appl. Microbiol.*, **3**, 1 (1957).

4. Kojic acid production by the heterocaryons

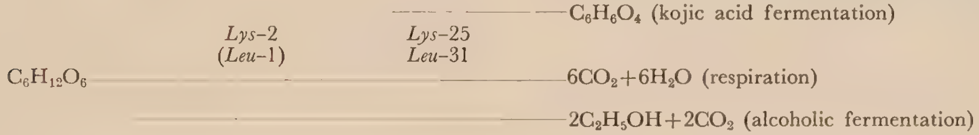
Kojic acid production by the heterocaryons between a kojic acid producer and a non-producer was studied as follows: First, the heterocaryons between *Lys*-22 and *Leu*-1 and those between *Lys*-22 and *Leu*-31 were cultivated in Czapek medium (minimal medium). When heterocaryotic mycelia developed into pellets, small hyphal fragments were separated aseptically and then inoculated into the malt extract medium in test tubes.

Kojic acid production was measured after 10 days. Figure 2 shows the distribution of acid production among the 40 test tubes gained from the parallel cultures of the heterocaryons and the parental strains. As seen in the figure, the heterocaryons between *Lys*-22 and *Leu*-1 show the same distribution pattern as one of the parental strains, *Lys*-22, while the heterocaryons between *Lys*-22 and *Leu*-31 show a different figure. A possible background for the different expression will be discussed later.

DISCUSSION

It is known in *Neurospora crassa*[8, 9] and *Saccharomyces cerevisiae*[10] that the metabolic activity via respiration could be abolished by mutation at either cytoplasmic factor or nuclear genes. In the present case, strain *Lys*-2, which is a slow grower in liquid or on a solid medium, accumulates ethylalcohol in a yield of 70 per cent of the theoretical value even under aerobic conditions, furthermore, the heterocaryons between the strain and a wild-type strain (*Leu*-23) give both component-type segregants upon conidiospore formation. It is due to these reasons that the author ventures to nominate the strain as a respiratory deficient mutant in spite of the incomplet analysis of the cytochrome system of the strain.

Kojic acid non-producers were very frequently obtained from strain *Leu*-23, but not from *Lys*-22. Though the reason why strain *Leu*-23 was unstable in regard to kojic acid production is not known, in any event, it is evident that the variation from a producer to a non-producer is a mutational event. In addition, the homo- and hetero-fermentative-type strains are concurrently kojic acid non-producers. Consequently, as a conclusion, the sites of genetic blocks which took place in each strain can be expressed as follows:


$C_6H_6O_4$ (kojic acid fermentation)
Lys-2
(*Leu*-1)
Lys-25
Leu-31
$C_6H_{12}O_6$
$6CO_2+6H_2O$ (respiration)
$2C_2H_5OH+2CO_2$ (alcoholic fermentation)

As mentioned before, the heterocaryons produced between a wild-type strain (*Lys*-22) and a hetero-fermentative-type strain (*Leu*-1) produced kojic acid in the same amount as one of the parental producer (*Lys*-22). On the other hand, the heterocaryons between a wild-type strain (*Lys*-22) and a respiratory-type strain (*Leu*-31) produced kojic acid in less quantities. This different expression can be explained as follows: That is, under the assumption that the three alternative pathways are competitive for sugar metabolism, the difference can be taken as a reflection of the dominance-recessive relationship underlying among the three metabolic pathways. In other words, it is probably on account of the suppresive action of respiratory nuclei that the production of kojic acid by the wild-type nuclei was affected by the co-existance of respiratory nuclei.

From a genetical point of view, two interpretations on the suppresive effect are pre-

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[8] M. B. Mitchell and H. K. Mitchel, *Proc. Natl. Acad. Sci.*, **38**, 442 (1952).

[9] M. B. Mitchel, H. K. Mitchel, and A. Tisseres, *ibid.*, **39**, 606 (1953).

[10] B. Ephurussi, Nucleo-Cytoplasmic Relation in Microorganisms. Clarendon Press (1953).

sumable. The first hypothesis is that the respiratory-type nuclei do take a selective advantage over the wild-type nuclei in tendency of reproduction. The second hypothesis is that the respiratory genes are dominantly fixed over oxidative fermentation genes. Both hypothesis could be accepted at the present stage of study, but as it was evidenced by one of the author's co-workers (Mr. K. Kiritani) that the ratio of two type nuclei in heterocaryons of *Asp. oryzae* is not constant throughout their growth stages, the second hypothesis seems to be more accountable.

In any event, the advantage-disadvantage or dominance-recessive relationship mentioned above should be evaluated in connection with experimental conditions. Accordingly, the conclusion drawn in the later part of this chapter does not rule out other possibilities. This is an inevitable disadvantage accompanying work of this kind.

[Bull. Agr. Chem. Soc. Japan, Vol. 22, No. 4, p. 238~242, 1958]

Germination Promotant for Plants Seed, produced by Microorganisms

Part IV. The Germination Promotant for Rice Plant Seeds, produced by *Streptomyces* sp. S–580. Isolation and Structure of Another Active Crystal (Factor–D).*

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Received March 25, 1958

A crystalline substance (Factor–D), by which the activity of Factor–E in promoting the germination of rice plant seeds could be increased synergistically, was isolated from the active crude syrup from which Factor–E was also isolated. This Factor–D has been proved by synthesis to be 1-methyl-2, 4-imidazoledione (1-methyl hydantoin). This substance has not been isolated from culture filtrates of any microorganisms.

INTRODUCTION

It has been already reported[1] that a neutral crystalline substance (Factor–E), which is effective for the germination of rice plant seeds at the dilution range of 10^{-5}–10^{-7}, was isolated from the active crude syrup obtained from the culture filtrate of *Streptomyces* sp. S–580. This substance was further proved by hydrolysis and synthesis to be L-prolyl-L-valine anhydride (diketopiperazine), a new diketopiperazine. In marginalia of that report the author has made reference that the activity of Factor–E could be increased synergistically by another crystalline substance isolated from the same active crude syrup. This substance, Factor–D, has been isolated by a silica-gel chromatography and proved by synthesis to be 1-methyl-2, 4-imidazoledione (1-methyl hydantoin). It may also be stated that Factor-

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* Presented at the Meeting of the Agricultural Chemical Society of Japan held in Tokyo, February 22, 1958.

[1] Y. Koaze, This Bulletin, **22**, 98 (1958).

D is solitary effective for the promotion of germination of rice plant seeds at the concentration range of 10^{-3}–10^{-4} M. Whereas, when it is used with Factor–E (in the concentration range of 10^{-4}–10^{-6} M), the synergistically effective concentration range of Factor–D was 10^{-5}–10^{-6} M. In 1913 1-methyl hydantoin had been isolated by D. Ackermann[2] from the culture filtrate of certain putrifying bacteria cultured in medium added with creatinine. However this substance has not been isolated from culture filtrates of any microorganisms grown on a conventional medium in the absence of creatinine.

EXPERIMETAL AND RESULTS

1) Isolation of Factor-D from the active crude syrup (A. C. S.).

Factor–D was isolated by a silica-gel chromatography from A.C.S. in the following manner. (a) 30 grm of dried silica (Mallinckrodt, 100 mesh) was thoroughly mixed with 22 ml of distilled water in a mortar and then mixed with 180 ml of pure chloroform to be gelatinized. (b) The silica-gel obtained was introduced into a glass tube with a diameter of 2.2 cm and 24 cm in height, (c) then, 1 grm. of A. C. S. dissolved in 1 ml of pure chloroform was charged into the gilica-gel column and was eluted at a flow rate of 1.5–2.0 ml/min. with each 100 ml of chloroform-*n*-butanol mixture saturated with distilled water (volume- percentages of *n*-butanol in the mixtures were as follows: 0, 5, 10, 15, 20, 30, 40 and 50, respectively). (d) Each 10 ml of the eluant was collected into a test tube and titrated with N/20 NaOH; the chromatograph obtained is shown in Fig. 1, and fractions of tubes No. 33–35 were combined and evaporated *in vacuo*. (e) Presently, a crystalline substance appeared in the pale-brown syrup, and then repeated crystallizations from ethanol gave colorless prisms (Factor–D) as shown in Fig. 2.

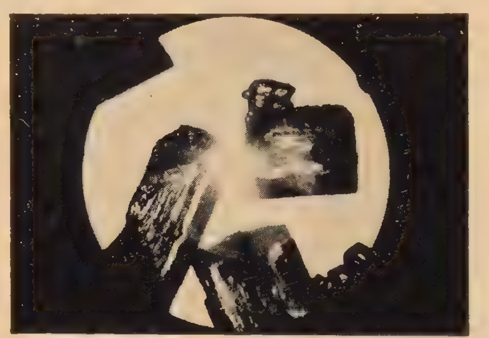

FIG. 2. Crystalline Form of Factor–D (from ethanol) (magnification ×80)

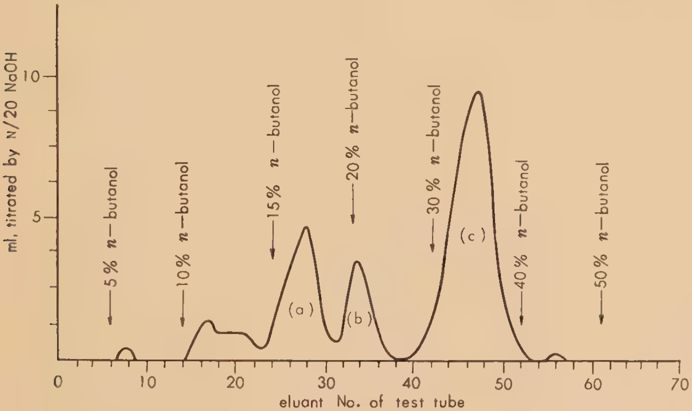

FIG. 1. A Silica-gel Chromatograph of the Active Crude Syrup (A. C. S.).

A silica-gel chromatograph of the active crude syrup (A.C.S.) obtained as follows: (a) 30 grm of dried silica (Mallinckrodt, 100 mesh) was mixed with 22ml of distilled water in a mortar thoroughly, then mixed with 180 ml. of pure chloroform to be gelatinized. (b) The silica-gel obtained was introduced into the glass tube with diameter 2.2 cm, height 24 cm, (c) then 1 grm of A.C.S. dissolved in 1 ml of pure chloroform was charged into the silica-gel column and was eluted at the flow rate of 1.5∼2.0 ml/min. with each 100ml. of chloroform-*n*-butanol mixture saturated with distilled water (volume-percentage of *n*-butanol in the mixture: 0, 5, 10, 15, 20, 30, 40 and 50). (d) Each 10ml. of eluant was collected into test tube and titrated with N/20 NaOH. Fraction (b) is Factor-D. It might be most probable that Fraction (a) and Fraction (c) were identified with fumaric acid and succinic acid respectively by means of paper chromatography.

2) Properties of Factors–D.

Factor-D is a colorless prism (derived from ethanol) having a weak acidic character, m. p. 157°C; easily soluble in acetone, alcohols and water, soluble in chloroform and ethyl acetate, very slightly soluble in benzene and ethyl ether, but insoluble in *n*-hexane and petroleum ether. Analytical data of this substance was as follows: *Anal.* Calcd. for $C_4H_6O_2N_2$: C, 42.1; H, 5.3; N, 24.6; mol. wt., 114.1; Found: C, 42.5; H, 5.5; N, 24.1; mol. wt. (performed

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[2] D. Ackermann, *Z. Biol.*, **62**, 208 (1913).

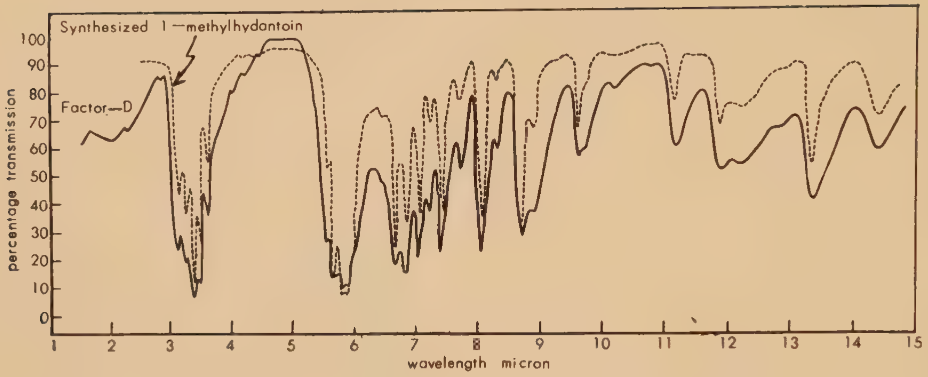

FIG. 3. The Infra-red Spectrum of Factor-D and Synthesized 1-Methyl Hydantoin in Nujol Mull.

by the micro-Rast method, dissolving in camphor), 109. Therefore, this substance was proved to have the empirical formula: $C_4H_6O_2N_2$. The UV-spectrum of this substance in methyl alcohol showed no significant absorption in the wave-length range of 220–400 mμ, but showed a strong end-absorption. The infra-red spectrum of this substance in Nujol mull is shown in Fig. 3. The ninhydrin reaction test to this substance was negative but the test with sodium nitroprusside3) was positive. Furthermore, by a paper chromatography (filter paper, Toyoroshi No. 50; solvent, *n*-butanol : acetic acid : water$=4:1:5$; ascending for twenty or twenty-four hours at room temperature; development of spots, a chlorine-starch method4) employed), this substance could be detected to have $R_F=0.62\pm0.06$.

3) Synthesis of 1-methyl-2, 4-imidazoledione (1-methyl hydantoin).

According to the method described by L. Baumann5), 1-methyl hydantoin was formed from urea and sarcosine which had been formed from methylamine hydrochloride and potassium cyanide in a formaldehyde solution.

As shown in Fig. 3, the infra-red spectrum of Factor-E is identical with that of synthesized 1-methyl hydantoin, furthermore on the paper chromatography, the R_F of the former is identical with that of the latter and a mixed melting point of the both gave no depression. Therefore, from these evidences, Factor-D has been proved to be 1-methyl-2,4-imidazoledione (1-methyl hydantoin).

4) Effect of Factor-D on the germination of rice plant seeds.

Fujisaka-No. 5 harvested in 1956 was used in this experiment. Conditions on the germination of rice plant seeds, determination of the activity in promoting the germination and preparation of the test solution were the same as described in the previous papers6,7). However, the test solutions used in this experiment were diluted with 10^{-4}M ammonium sulfate solution. As indicated in Table I, Factor-D, alone, showed a promoting effect on the germination of rice plant seeds in the concentration range of $10^{-3}-10^{-4}$ M. Furthermore

TABLE I. EFFECT OF FACTOR-D ON THE GERMINATION OF RICE PLANT SEEDS

Nine days after start of experiment incubated in dark condition at 18°C, stem and root elongation, prophyll and crown root formation ratio were determined. Also six days after, interim-germination ratio was determined. And Factor-D was diluted with 10^{-4} mol ammonium sulfate aq. solution as test solutions. (refer to the previous papers1,6,7))

	Control1)	10^{-3}mol	10^{-4}mol	10^{-5}mol	10^{-6}mol
stem cm (%)	1.56	1.89 (21)2)	2.06 (32)	1.71 (10)	1.67 (7)
root cm (%)	3.86	5.09 (32)	5.55 (44)	4.04 (5)	4.62 (20)
prophyll formation ratio (%)	25	80	75	30	40
crown root formation ratio (%)	10	70	70	25	35
interim-germination ratio (%)	35	65	70	30	40

NOTES

1) Treated with 10^{-4}mol ammonium sulfate aq. solution without Factor-D.

2) Figures in the brackets show the promoting ratio against control.

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3) F. Feigl, Spot Tests In Organic Analysis (1956), p. 223.

4) H. N. Rydon and P. W. G. Smith, *Nature*, **169**, 922 (1952).

5) L. Baumann, *J. B. C.*, **21**, 565 (1915).

6) Y. Koaze et al., *J. Agr. Chem. Soc. Japan*, **31**, 338 (1957).

7) Y. Koaze, This Bulletin, **22**, 91 (1958).

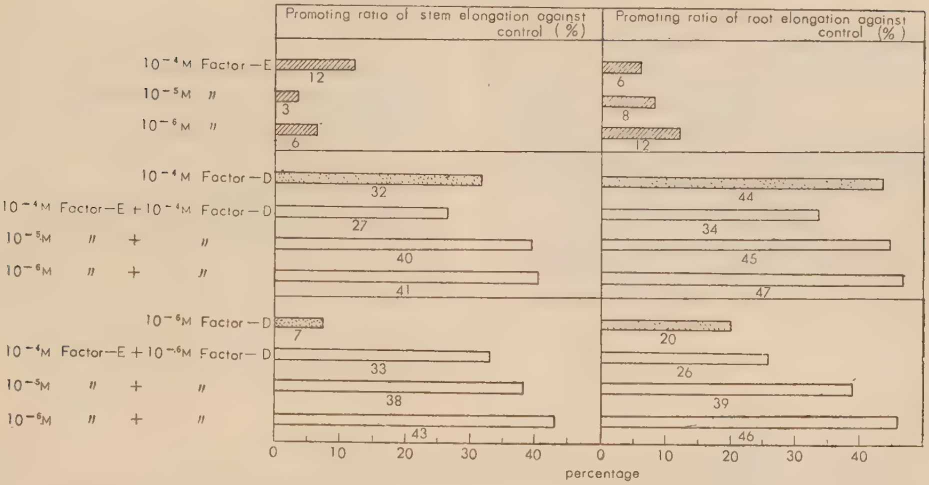

FIG. 4. Synergistic Action of Factor-E and Factor-D in Promoting the Germination of Rice Plant Seeds.
(I) Effects on Promoting Ratio of Stem and Root Elongation Against Control.

Nine days after start of experiment incubated in dark condition at 18°C, stem and root elongation were determined. Factor-D and E were diluted with 10^{-4} mol ammonium sulfate aq. solution as test solutions. (refer to the previous papers[1,6,7])

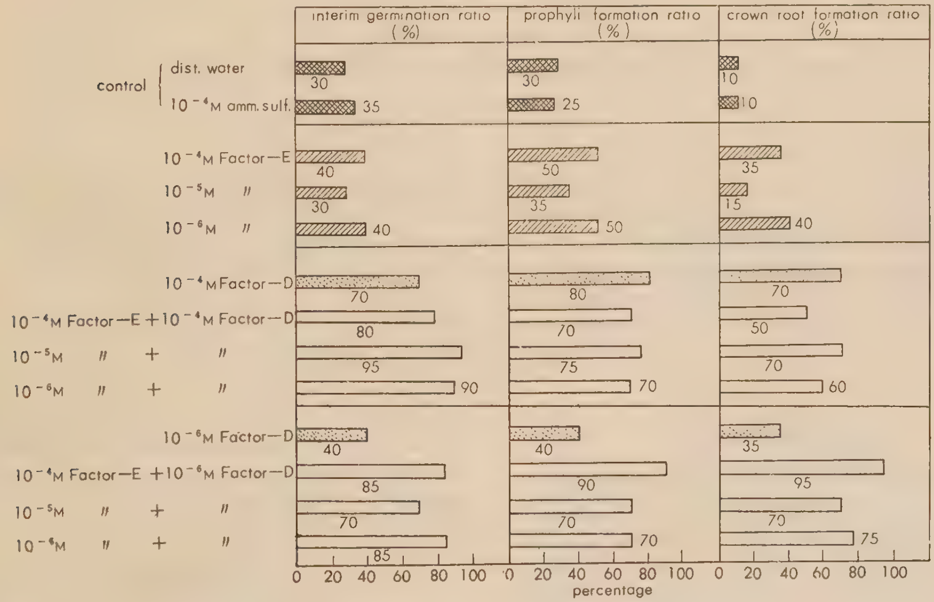

FIG. 5. Synergistic Action of Factor-E and Factor-D in Promoting the Germination of Rice Plant Seeds.
(II) Effects on Interim-germination Ratio, Prophyll and Crown Root Formation Ratio.

Nine days after start of experiment incubated in dark condition at 18°C, prophyll and crown root formation ratio were determined. Also six days after, interim-germination ratio was determined. And Factor-D and Factor-E were diluted with 10^{-4} mol ammonium sulfate aq. solution as test solutions. (refer to the previous paper[1,6,7])

Factor–D showed, synergistically, a promoting effect on the germination in the concentration range of 10^{-5}–10^{-6} M with 10^{-4}–10^{-6}M of Factor–E. For example as shown in Figs. 4 and 5, mixtures of 10^{-6} M Factor–D and 10^{-4}–10^{-6} M of Factor–E were synergistically effective for promoting germination, however, activities of mixtures of 10^{-4} M Factor–D and the same concentrations of Factor–E were found to be similar to those in case of 10^{-4} M Factor–D alone. (These activities were less than that of the culture filtrate of *St.* sp. S–580. Consequently, studies concerning this fact are now in progress.)

Acknowledgements The author wishes to express his sincere thanks to Prof. K. Sakaguchi, Univ. of Tokyo for his guidance throughout this work. Thanks are also due to Prof. Y. Sumiki and Prof. T. Asai, Univ. of Tokyo for their kind advice and helpful suggestions. Micro-analysis and the infra-red spectrum were carried out in this department.

[Bull. Agr. Chem. Soc. Japan, Vol. 22, No. 4, p. 242~248, 1958]

Studies on the Opalescence in Sodium-caseinate Solution Developed by the Milk Coagulating Enzymes

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Received February 7, 1958

Both rennet and pepsin are used as a coagulant in cheese-making process. The proteolytic action of these two milk-coagulating enzymes was studied. In this report, results of the experiments on the development of the opalescence in sodium-caseinate solution by two enzymes are reported. Some properties of the opalescent materials and the change in the distribution of nitrogen and phosphorus are also included.

These experiments show the difference of the mode of proteolytic action on casein by rennet and by pepsin, which may be concerned with the ripening of cheese.

INTRODUCTION

Rennet used for coagulating milk in cheese making, also hydrolyses casein and its proteolytic action may be significant for the ripening of cheese. Pepsin instead of rennet was also used as a coagulant in cheddar cheese making.[1] Experiments were conducted on the proteolytic action of milk-coagulating enzymes in order to clarify the change of casein during cheese-ripening.

This report presents the results of the experiments on the development of the opalescence in sodium-caseinate solution by the action of rennet and pepsin.

EXPERIMENTAL

Materials and methods

Casein was prepared from unpasteurized cow's skim milk by the method of Hipp.[2] α-Casein was prepared by the method of Warner[3] and β-casein by the method of Hipp[4] with the use of urea, specifically. Both of them were electrophoretically pure at pH 7.6.

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[1] T. Yamamoto, K. Takahashi, G. Takema and M. Yoshino, *Bull. Natl. Inst. Agr. Sci., Japan.* Ser. G. No. **10**, 21 (1955).

[2] N. J. Hipp, M. L. Groves, J. H. Custer and T. L. McMeekin, *J. Am. Chem. Soc.*, **72**, 4928 (1950).

[3] R. C. Warner, *J. Am. Chem. Soc.*, **66**, 1725 (1944).

The enzymes used were Hansen's Rennet Tablet and Mikuni's pepsin preparation. Hydrolysis was carried out at pH 6.5 on 2.5% sodium caseinate solution at 35°C.

RESULTS AND DISCUSSION

1) Relation between the opalescence and hydrolysis of casein

Casein was hydrolysed by various concentrations of rennet and pepsin. The change of the opalescence in the reaction mixture measured with time is shown in Fig. 1. The change of the optical density of casein solution due to the development of the opalescence by rennet or pepsin was measured with a Model 9 Coleman Nephocolorimeter, using water as the reference medium without using a filter.

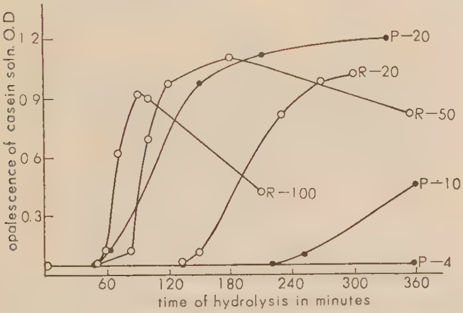

FIG. 1. Development of the Opalescence in Sodium-caseinate Solution by Various Concentrations of Rennet (–○–) or Pepsin (–●–).

R and P represent rennet and pepsin respectively and figures show mg of enzymes added to 10 c.c. of casein solution.

The concentration of rennet and pepsin was adjusted so as to produce the same amount of non-casein nitrogen as shown in Fig. 2, in which each pair of R-100 and P-20, R-50 and P-10, and R-20 and P-4 gave the same amount of non-casein nitrogen. In this place, R stands for rennet, P for pepsin, and the figures represent mg. of enzymes added to 10 c.c. of casein solution. The hydrolysis of casein by rennet or pepsin was measured, by means of removing unhydrolysed casein from the aliquots of the reaction mixture by the addition of a pH 4.6 acetate buffer solution. Nitrogen in the filtrate was determined by the semi-micro Kjeldahl method, representing percentage against the total nitrogen.

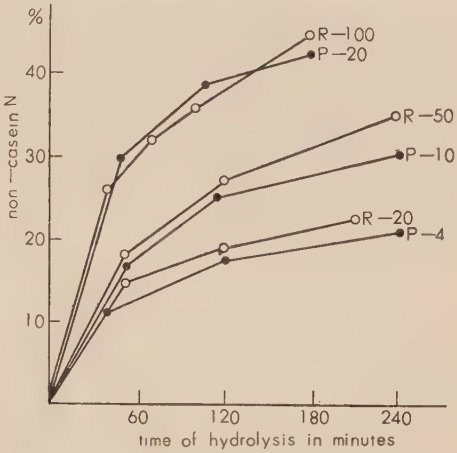

FIG. 2. Hydrolysis of Casein by Various Concentrations of Rennet (–○–) or Pepsin (–●–).

As a whole, the opalescence in casein solution developed more rapidly with rennet, than with pepsin which caused the same extent of hydrolysis as rennet. This difference in the development of opalescence was specially notable when R-20 was compared with P-4; that is, while R-20 developed considerable opalescence, P-4 developed entirely no opalescence.

When the concentration of rennet was increased, the opalescence developed rapidly to its maximum and also disappeared rapidly, thereafter. At low concentrations of rennet, both the development of the opalescence and its disappearance were slow. In the case of pepsin, which hydrolyses casein to the same extent as rennet, the development of the opalescence was either delayed, or entirely not observed. However, when a larger

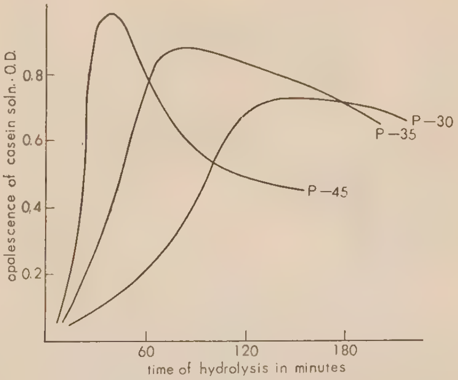

FIG. 3. Development of the Opalescence in Casein Solution by a Larger Amount of Pepsin.

[4] N. J. Hipp, M. L. Groves, J. H. Custer and T. L. McMeekin, *J. Dairy Sci.*, **35**, 272 (1952)

amount of pepsin was used, both development and disappearance of the opalescence were rapid. (Fig. 3)

It is understood, therefore, that rennet hydrolyses casein and produces the non-casein nitrogen and the opalescent material simultaneously. Conversely, pepsin produces the opalescent material in the hydrolysis process after pepsin hydrolyses casein to an extent that it liberates more non-casein nitrogen than rennet.

Furthermore, it was observed in Fig. 1 that a part of the casein molecule was supposed to be degraded by the rennet action and to produce the opalescent material which decreased by successive rennet action. The opalescent material produced by the action of pepsin was not decreased easily by pepsin.

Percentages of non-casein nitrogen of the total nitrogen taken at the moment when the opalescence started to develop were 32 %, 22 %, 20 % in R-100, R-50 and R-20 and 33 %, 29 % in P-20 and P-10 respectively. No direct relation was observed between the development of the opalescence and hydrolysis of casein but, in producing the opalescence a difference was observed between the rennet action and the pepsin action.

2) Effect of heat treatment

The effect of preliminary heating of casein solution on the development of the opalescence was studied.

Sodium-caseinate solution was heated in a sealed glass-tube for sixty minutes at different temperatures of 80°C, 100°C and 120°C. This heat treatment itself had no effect in increasing either the opalescence or digestibility of casein solution. The results are shown in Fig. 4. The development of opalescence in sodium-caseinate solution by an enzyme action seems to be accelerated by preliminary heat treatment. The heat treatment had more effect on the opalescence developed by pepsin than that by rennet.

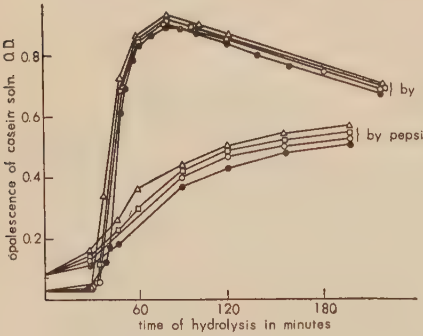

FIG. 4. Effect of Preliminary Heating of Casein Solution on the Development of the Opalescence by Rennet or Pepsin.

Heating was carried out for 60 minutes at 80° (—○—), 100° (—△—) or 120° (—□—). Control experiment is shown by —●—.

3) Effect of salt

Effect of the presence of sodium chloride or sodium sulfate on the opalescence in the reaction mixture was studied. The opalescence produced by rennet or pepsin in sodium caseinate solution containing 0.75 %, 2.25 % and 3.75 % of salt respectively was measured. The results are shown in Fig. 5.

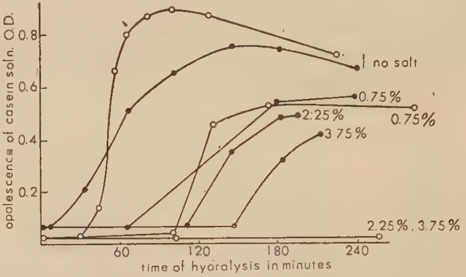

FIG. 5. Effect of the Presence of NaCl or Na_2SO_4 on the Development of the Opalescence by Rennet (—○—) or Pepsin (—●—).

The development of opalescence by rennet was retarded by the presence of 0.75 % salt, and was completely inhibited in the presence of 2.25 % salt. The development of the opalescence by pepsin was also slow in the presence of salt, but could still be observed in the presence of 3.75 % salt which inhibited the development of the opalescence by rennet. There was no difference between the inhibitory effect of sodium chloride and that of sodium sulfate.

As the salt concentration used here showed no influence on the proteolytic action of rennet or pepsin as shown in Table I, the change in the opalescence was considered to be due to the dissolution of the opalescent material contained in the salt solution. This was ascertained by the appearance of opalescence after a dialysis of casein solution which contained 3.75 % sodium chloride, and remained transparent after the rennet action. Therefore, the opalescent material

TABLE I. THE EFFECT OF SALT ON THE PROTEOLYSIS OF CASEIN BY RENNET OR PEPSIN.

The degree of proteolysis was shown as the increase of non-casein N in % of total N after sixty minutes' proteolysis.

Salt (NaCl*) %		0	0.75	2.25	3.75
Increase of non-casein N in %	Rennet	18.2	18.4	18.4	18.3
	Pepsin	32.3	32.7	32.6	32.4

* The experiment on Na_2SO_4 also gave the same results as NaCl

assumes a globulin-like nature in its solubility. It also dissolves in alkali.

4) Opalescence in the fractionated casein solution

The action of rennet or pepsin on α- and β-casein was studied. Both rennet and pepsin developed opalescence in α-casein solution, but neither developed opalescence in β-casein solution. (Fig. 6)

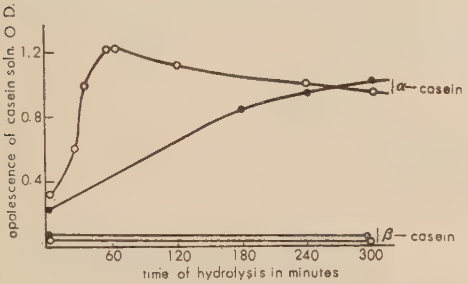

FIG. 6. Development of the Opalescence in Fractionated Casein Solution by Rennet (—○—) or Pepsin (—●—).

Christensen[5] reported that in α-casein solution opalescence is observed only with chymotrypsin, and in β-casein solution with chymotrypsin, trypsin and plasmin. He also reported that a temporary increase of viscosity is observed in β-casein solution when trypsin or plasmin is used.

In the present experiment, the action of rennet or pepsin caused only a gradual decrease in viscosity of both α- and β-casein solutions. Viscosity was measured by an Ostwald viscosimeter at 35°C. Rennet and pepsin produced the rise in the viscosity of casein solution at pH 2, forming gelatinous material in the reaction mixture, but at pH 6.5 gelatinous material was not formed. This fact suggests the difference of the type of actions of these two enzymes at two separated pH ranges.

5) Fractionation of hydrolysed casein solution

In order to measure the liberation of nitrogen and phosphorus from casein by the milk-coagulating enzymes, the separation of unhydrolysed casein from hydrolysis products is necessary.

Based on the ground that the opalescent casein solution is composed of unhydrolysed casein, opalescent material, and soluble products, the opalescent material should be removed or dissolved by appropriate means before unhydrolysed casein is precipitated at pH 4.6.

As the neutral salt dissolves the opalescence to the extent as shown in Fig. 5, a sufficient concentration of salt necessary to make the opalescent casein solution transparent had to be sought for. The opalescence of the casein solution by rennet dissolved completely in the presence of 2% sodium chloride, while most of the opalescence produced by pepsin remained undissolved. (Fig. 7)

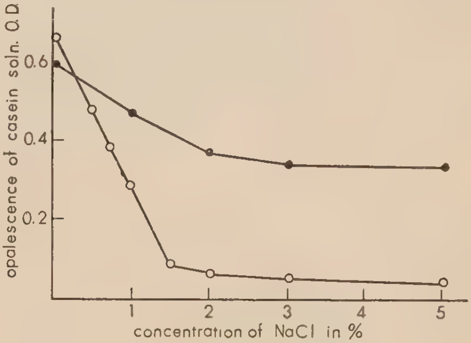

FIG. 7. Dissolution of the Opalescent Material in Various Concentration of NaCl.

—○— the opalescence by rennet
—●— the opalescence by pepsin

Raising the salt concentration to more than 5% resulted in the salting out of casein. It follows that neutral salt was found to be inadequate for this purpose. These results also proved that the opalescent materials produced by rennet and pepsin are both different substances.

The opalescent material was precipitated by a centrifugation for sixty minutes at 12,000 r.p.m. in a low temperature range of 0°~−2°C, giving a clear

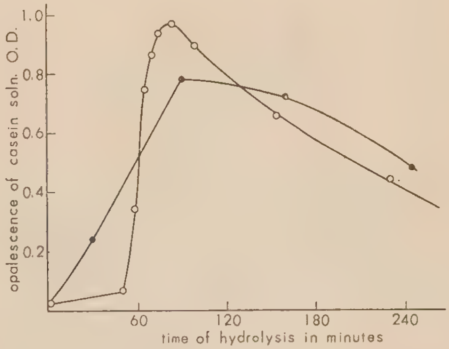

FIG. 8. Change of the Opalescence by Heating at 80° for 10 Minutes.

—○— before heating —●— after heating

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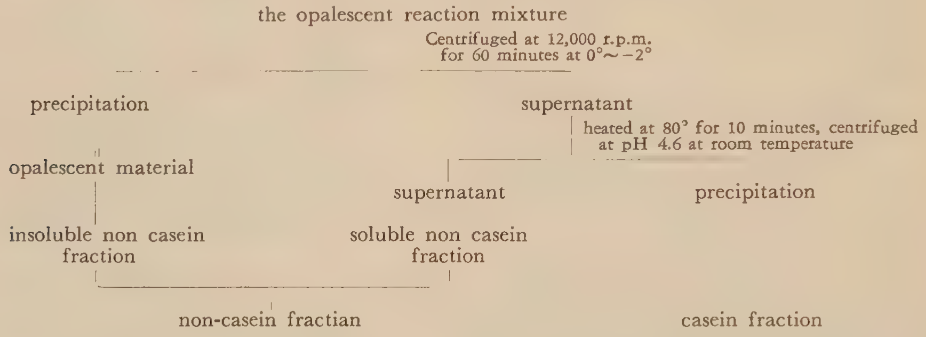
[5] L.R. Christensen, *Arch. Biochem. Biophys.*, **53**, 128 (1954).

supernatant. Heating was avoided to cease the enzyme action because heating at 80°C for ten minutes caused changes in the opalescence of casein solution. (Fig. 8)

Samples were removed from the reaction mixture at appropriate time intervals, and the opalescence was measured and kept frozen at −20°C prior to centrifugation. Freezing at −20°C did not affect the opalescence of the casein solution. After the centrifugation the supernatant was heated at 80°C for ten minutes, and the unhydrolysed casein was precipitated at pH 4.6 at room temperature. The outline of the method of fractionation employed is shown in Fig. 9.

FIG. 9. Method of Fractionation of the Opalescent Reaction Mixture


the opalescent reaction mixture
Centrifuged at 12,000 r.p.m. for 60 minutes at 0°∼−2°
precipitation
supernatant
heated at 80° for 10 minutes, centrifuged at pH 4.6 at room temperature
opalescent material
supernatant
precipitation
insoluble non casein fraction
soluble non casein fraction
non-casein fractian
casein fraction

6) Determination of the nitrogenous content in each fraction

Changes of the nitrogenous contents in each fraction in the process of hydrolysis obtained by the method shown in Fig. 9 were determined. The results are shown in Fig. 10.

The nitrogenous content of the opalescent material produced by rennet or pepsin at the peak of the opalescence amounted to about 25% of the total casein nitrogen, which decreased as the hydrolysis reaction proceeded, to 5% with rennet and to 15% with pepsin. This result shows the difficulty of digestion of the opalescent material by pepsin.

Nitrogen of the opalescent material produced by rennet reached 78% of non-casein nitrogen at the peak of opalescence, which gradually decreased to 10%. When pepsin was used, nitrogen of the opalescent material reached only 31% of non-casein nitrogen and decreased to 16%.

The difference of ratio of the opalescent material nitrogen to non-casein nitrogen shows that when casein is hydrolysed by rennet more opalescent material is produced than by pepsin.

As indicated in Fig. 10, when rennet was used an increase of the soluble non-casein nitrogen was observed after the opalescent material, once reaching the peak,

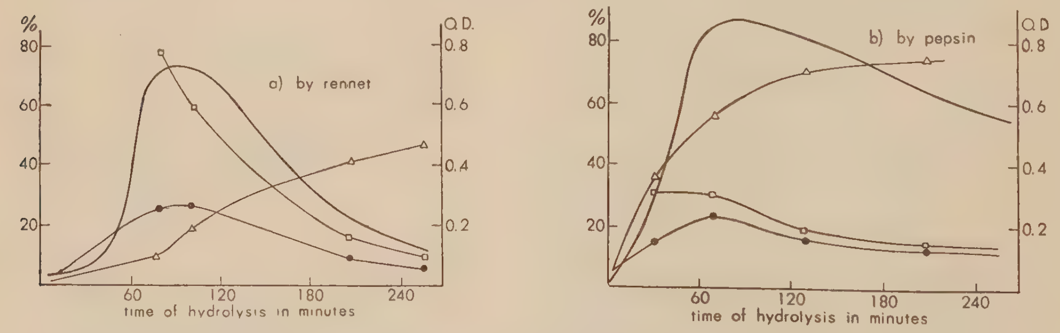


FIG. 10-1. FIG. 10-2.

FIG. 10. N-Distribution in Each Fraction.

—— Opalescence of Casein Soln. (O.D.)
—●— N of the Opalescent Material in % of Total Casein N.
—△— N of Soluble non-Casein Fraction in % of non-Casein N.
—□— N of the Opalescent Material in % of non-Casein N.

started to decrease, and in the case of pepsin the opalescent material and soluble non-casein nitrogen were produced simultaneously.

Certain specific bonds are supposed to be split during the enzymatic stage of coagulation. It was apparent that no direct relation between the coagulation and the opalescence exists, since in the present experiments, though the required amount of rennet to coagulate casein solution instantly in the presence of calcium ions was added, opalescence developed some time after addition of rennet. In other words, the opalescent material is not produced directly from casein molecule, but is a secondary product of the hydrolysis of casein. The splitting of bonds, which is supposed to have occurred during the development of opalescence, is not related to the coagulation reaction. The same fact is confirmable with pepsin.

7) Determination of the phosphorous content in each fraction

Changes of the phosphorous content in each fraction in the hydrolysis process obtained by the method shown in Fig. 9 were determined. Phosphorus was determined by Allen's method modified by Nakamura.6)

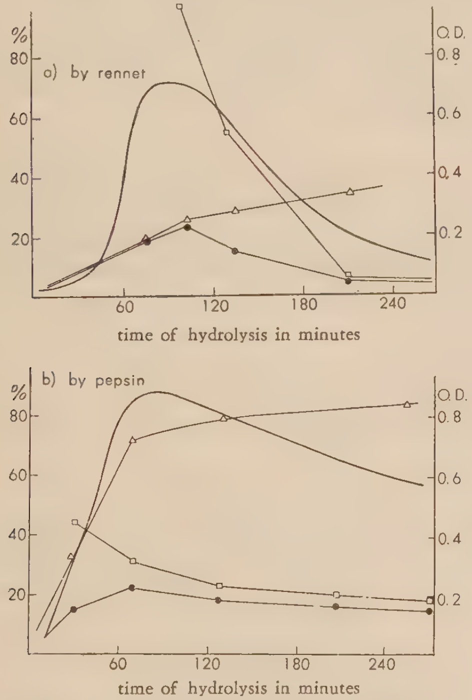


FIG. 11. P-Distribution in Each Fraction.

—— Opalescence of Casein Soln. (O.D.)
–●– P of the Opalescent Material in % of Total Casein P.
–△– P of Soluble non-Casein Fraction in % of non-Casein P.
–□– P of the Opalescent Material in % of non-Casein P.

Changes of the phosphorous content in each fraction were similar to those of the nitrogenous content. (Fig. 11) Percentages of the phosphorous content in the opalescent material at the peak of opalescence were 23 % and 21 % of the total phosphorous when rennet and pepsin were used respectively. The percentage proportion of the phosphorous content to the phosphorus in the non-casein fraction was almost 100 % with rennet, and 33 % with pepsin.

8) N/P ratio of each fraction

In the hydrolysis condition chosen, 40 % of the original casein remained as the unhydrolysed fraction when rennet was used, and 8 % remained when pepsin was used.

The N/P ratio of the original casein was 15.8 and the N/P ratio of the casein fraction decreased as the reaction proceeded. (Fig. 12) At an early stage of hydrolysis, there was a quite rapid decrease caused by pepsin, and a gradual decrease caused by rennet. The casein fraction is considered to be also hydrolysed to a certain extent.

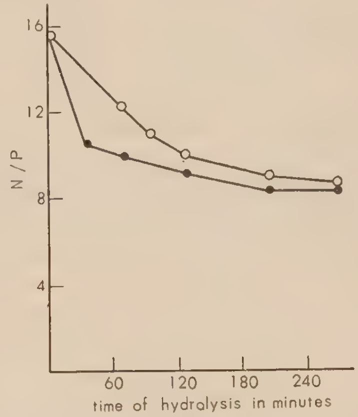


FIG. 12. Change of N/P Ratio of the Casein Fraction.

–○– hydrolysis by rennet.
–●– hydrolysis by pepsin.

It has been well known that after the hydrolysis of casein by trypsin or pepsin, a phosphopeptone which has an N/P ratio of around 5 or less is obtained. The opalescent material obtained in the present experiments shows at the peak of the opalescence, an N/P ratio of 23 by rennet and 18 by pepsin; that is, the phosphorous content of the opalescent material is lower than that of the original casein.

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6) M. Nakamura, *J. Agr. Chem. Soc. Japan*, **24**, 1 (1950).

In the casein solution at pH 2, a gelatinous material was formed with pepsin or rennet. The gelatinous material formed by pepsin is easily precipitated by centrifugation, but the gelatinous material by rennet is not easily precipitated, and contains much liquid. These gelatinous materials did not disappear as the enzymatic action proceeded. The N/P ratios of these gelatinous materials were 6.6 by rennet, and 3.4 by pepsin, which were also different from the N/P ratio of the opalescent material.

CONCLUSION AND SUMMARY

The development of the opalescence in casein solution by the action of rennet or pepsin was investigated. When the concentrations of two enzymes are adjusted so that they have the same hydrolytic activity, rennet develops more opalescence than pepsin, and as the reaction continues, the opalescence decreases when rennet is used while the opalescence developed by pepsin remains almost unchanged.

The preliminary heating of casein solution has an accerelating influence on the development of the opalescence caused by these enzymes.

In consideration of the fact that there is such difference of solubility in neutral salt solution, the opalescent material produced by two enzymes are different from each other, and suggest the difference of the mode of action between the two enzymes.

Both rennet and pepsin develop opalescence in α-casein solution, but not in β-casein solution.

At the peak of the opalescence in the hydrolysis of sodium caseinate solution by rennet, the amount of nitrogen in the opalescent material reaches 78% of the total non-casein nitrogen, while when pepsin is used only 31% is attained. It is understood, therefore, that casein is hydrolysed by rennet through the opalescent material as its intermediate. The difference of hydrolytic action of rennet and pepsin on casein may lead to a new interpretation for cheese-making, when pepsin is used as a milk coagulant in place of rennet.

The change of the phosphorous content in each fraction is similar to that of the nitrogenous content. The gradual decrease of the N/P ratio in the casein fraction indicates that this fraction is hydrolysed to a certain extent. The opalescent material is not identical with phosphopeptone.

The author wishes to express his sincerest thanks to Prof. Rinjirô Sasaki, Department of Agricultural Chemistry, University of Tokyo, for his kind guidance throughout this work.

[Bull. Agr. Chem. Soc. Japan, Vol. 22, No. 4, p. 249~255, 1958]

Chemical Studies on the Autolysis of Meats

Part VII. On the Chemical Changes of Myosin B during aging of Meats.

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Received April 4, 1958

In order to study whether or not myosin B has been altered during the aging of meats, experiments were carried out on salting out, viscosity, ATP-sensitivity, and ATPase of myosin B solution.

Chemical properties of myosin B were found to be altered during the aging process of meat, especially AMS, a member of myosin B (thus named by one of authors) resulted in a high ratio of myosin in its constitution, and seemed to come to resemble the properties of myosin during the process.

In previous reports,[1,2] myosin, actin, myosin B, and sarcoplasmic proteins were determined quantitatively during the aging of meats, from which it was shown that the difference in the chemical natures of myosin-, and myosin B-ATPase during the aging process is dependent on the chemical changes of these proteins during the process.

As intact myosin B still remained in meats even after the meats had undergone the maximum of rigor mortis, and moreover the chemical nature of myosin B-ATPase has been altered during the aging process, it was interesting to study on the chemical natures of myosin B isolated from meats during the aging process to elucidate the mechanisms of meat tenderness subsequent to rigor motis.

In this report, salting out patterns, viscosity changes, and the effect of added adenosine triphosphate (ATP) on viscosity, i.e., ATP-sensitivity, of myosin B from meats during the aging process are presented.

EXPERIMENTAL

Methods. Preparation of myosin B from meat materials: Two kinds of myosin B, AMS and AML, given such names by one of the authors were prepared in the same manner as previously reported[1] from the following meats during the aging process (at about 0° for 12 days): rabbit (variety, Japanese white, body weight 3 kg, female, longissimus dorsi), veal (variety, Holstein, one year old, female, part-neck muscle), cow (Japanese Black Breed of Cattle, seven years old, female, part-neck muscle), and horse meat (variety, unknown, eight years old, castrated, part-diaphragm).

The name AMS was given to myosin B which is obtained by extracting meats for a short period with 0.6 M potassium chloride solution and then precipitated by dilution to an ionic strength of 0.1 with glass redistilled water ("dilution method"). AML is another kind of myosin B, extracted with the Weber-Edsall solution, for an extended period from the residue remaining after extraction of AMS, and refined by the "dilution method" as described above.

Salting out: The following experiments on salting out of myosin B were carried out according to the procedure of Yagi et al.[3] An amount of one-tenth to two gram of ammonium sulfate was added to each 5 ml. of myosin B solution in 0.6 M potassium chloride (1 gN/l), and after being allowed to stand over night at 0°, each solution was centrifuged and then filtered (Toyo filter paper, No. 3C). Optical density at 275 mμ of each filtrate was measured with a Beckman model D.U. spectrophotometer.

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[1] M. Fujimaki and Y. Nakajima, *J. Agr. Chem. Soc. Japan* (in press).

[2] M. Fujimaki, *J. Agr. Chem. Soc. Japan* (in press).

[3] K. Yagi et al., presented at the IXth Symposium on Enzyme Chemistry (1956).

Viscosity: Viscosity of the myosin B solution in potassium chloride was measured at 1° with an Ostwald viscosimeter. The intrinsic viscosity $[\eta]$ was calculated from the relative viscosity η_{rel} as follows:

$$[\eta]=(\eta_{rel}-1)/C,\quad C=\text{myosin B g/l}$$

ATP-sensitivity: To 5 ml. of myosin B solution in potassium chloride were added 0.25 ml of 2×10^{-2} M magnesium chloride solution and 0.5 ml of 1 % neutral sodium ATP solution. After it was mixed thoroughly, the relative viscosity $\eta_{rel(ATP)}$ was determined.

The ATP-sensitivity was calculated from the following equation.[4]

$$\text{ATP-sensitivity}=(\log\eta_{rel}-\log\eta_{rel(ATP)})/\log\eta_{rel(ATP)}\cdot100\ \%$$

ATPase-activity: Activity of ATPase was measured as previously reported,[2] and the reaction mixture which contained a final-volume of 3.0 ml, composed of 1.0 ml of 0.04 M veronal or 0.1 M citrate buffer solution, 0.5 ml of 6×10^{-2} M $CaCl_2$, 0.5 ml of 1.2 M KCl, 0.5 ml of myosin B solution in potassium chloride and 0.5 ml of 5×10^{-3} M neutral sodium ATP solution was incubated at 25°. At measured intervals of time, to aliquotes of the reaction mixture was added 1.0 ml of 10 % trichloroacetic acid solution and the protein precipiated was filtered off through a dry filter paper. The amount of inorganic phosphorus liberated in 1 ml of the filtrate was determined colorimetrically using a modification of the Allen method.[5]

One unit of ATPase was defined as the amount of enzyme which liberate 1 γ of inorganic phosphorus under the above conditions.

RESULTS AND DISCUSSION

Salting out: The results on salting out of myosin B solution are presented in Figs. 1 and 2. As shown in these figures, the optical densities of the AMS solutions from veal and

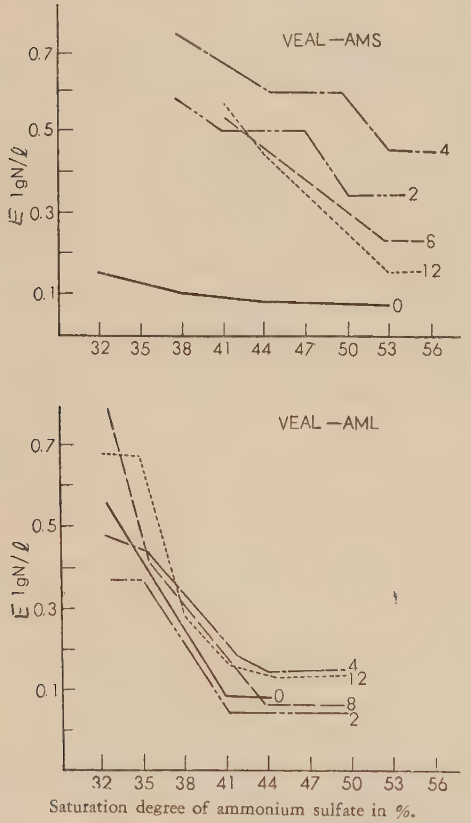

FIG. 1. Salting out Curves of Veal Myosin B Solution.

Numbers in Fig. show days of aging.

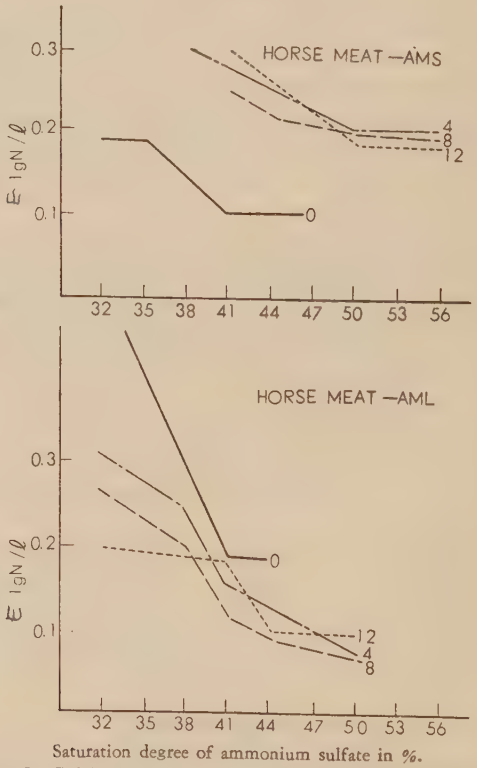

FIG. 2. Salting out Curves of Horse Meat Myosin B Solution.

Numbers in Fig. show days of aging.

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[4] W. Hasselbac~~k~~h and G. Schneider, *Biochem. Z.*, **321**, 462 (1951).

[5] M. Nakamura, *J. Agr. Chem. Soc. Japan*, **24**, 1 (1950).

horse meat obtained immediately after slaughter were very small as compared with those of AMS solutions from aged meats.

As the concentration of ammonium sulfate in the solution increased, salting out curves of AMS declined and turned to show a flat line.

The flat region was obtained by 38–41% saturated ammonium sulfate with veal AMS immediately after slaughter, by 50% after 2 days, and by 53% after 4 to 12 days of aging.

As it may be seen the concentration of ammonium sulfate in the salting out of AMS has been altered with the aging of meats, the maximum precipitating region of AMS seemed to approach that of myosin.

In veal AML, the maximum precipitating region was obtained by 41% saturated ammonium sulfate, immediately after slaughter and the following 2 days, by 44% after 4 to 12 days of aging. As observed in the case with AMS, the concentration of ammonium sulfate in salting out of AML has been shifted toward higher concentrations with the aging of meat.

Similar results were obtained with the salting out of myosin B of horse meat as it is seen in Fig. 2.

These results, in which the concentration of ammonium sulfate in the maximum precipitating region of myosin B has been raised with the aging of meats, seem to show changes of constitutional structure in myosin B with aging. For example, AMS from unaged meat seems to be different from the AMS of meats aged for 4–12 days, and AMS has a maximum precipitating region at a higher concentration of ammonium sulfate than AML. Therefore, from the results on salting out of myosin B, chemical differences of two kinds of myosin B will be obvious.

Viscosity: Intrinsic viscosity of myosin B in potassium chloride solution prepared from rabbit, veal, cow, or horse meat is presented in Table I. From the table, it is obvious that the intrinsic viscosity of AMS is lower than that of AML, irrespective of the kind of meats tested.

TABLE I

INTRINSIC VISCOSITY OF MYOSIN B SOLUTION.

Kinds of animal meats	Aging period (Days)	Intrinsic Viscosity	
		AMS	AML
Rabbit	0	0.27	0.50
	4	0.43	0.98
	8	0.17	0.60
Veal (Holstein Kind)	0	0.19	0.35
	2	0.13	0.72
	4	0.13	0.39
	8	0.14	0.51
	12	0.13	0.62
Veal (Japanese Black Breed of Cattle)	0	0.21	0.46
Cow meat	0	0.06	0.29
	2	0.04	0.28
	4	0.08	0.39
	8	0.06	0.37
Horse meat	0	0.26	0.45
	4	0.24	0.44
	8	0.32	0.54
	12	0.18	0.42

The intrinsic viscosities of AMS and AML from rabbit gave maximum values at about 4 days of aging, that is, at the time of maximum rigor. Similar results were obtained in the changes of intrinsic viscosities of horse-myosin B solution and the AML of veal and cow meats. These changes occurring in intrinsic viscosity of myosin B during the aging process of meats are therefore considered to indicate changes of constitutional structure in myosin B proceeding with the aging of meats and have some influence upon rigor mortis and the rigor off of meats.

On the other hand, the intrinsic viscosity of myosin B solution from different kinds of animal meats is presented in Table I from which it is observed that the intrinsic viscosity of myosin B from rabbit is very high, and that from the cow meat very low. Consequently, the difference of water binding capacity of meat depending on the kinds of animals may be partly attributed to the differences of intrinstic viscosity.

Also, from reports that the intrinsic viscosity of actomyosin (equivalent to myosin B referred in this paper) solution from fish muscles

(Yellow-striped rockfish) decreases after one day of aging at 0°,[6] chemical changes in muscles of fish post mortis are supposed to proceed more rapidly than in animal meat.

ATP-sensitivity : In aged meat it is important to determine the content of actomyosin (myosin B) and the ratio of myosin to actin in actomyosin.[4] The intrinsic or specific viscosity of myosin B is dependent on the relative ratio of actin to myosin in actomyosin, and when the ratio was 1:2.5, specific viscosity was at its highest.[7] When ATP is added to myosin B in potassium chloride solution at an appropriate concentration, viscosity decreases, the depression being maximum when the specific viscosity is the highest. Likewise as the ratio of actin to myosin in actomyosin differs a difference in the depression of viscosity is also observed. For example, when the content of actin in actomyosin is high, the depression of viscosity is large, while on the contrary, when the content of myosin is high, depression is small. Consequently, the degree of the depression of viscosity indicates the changes in the constitutional structure of myosin B.

Results on ATP-sensitivity is presented in Table II. As can be observed in the table, ATP-sensitivity of myosin B prepared from various kinds of meat was higher in AML than in AMS. This result was already suggested by one of the authors in a previous paper.[1] Moreover, Yagi et al. reported that when myosin B was extracted for an extended period, ATP-sensitivity of resulting myosin B assumes a high value.[8] ATP-sensitivity of AMS prepared from rabbit and horse meat showed the highest value after 4 to 8 days of aging which decreased thereafter, and in case of AMS from veal and cow meat a decrease was observed only during the aging process. Consequently, ATP-sensitivity became lower with the progress of aging, and gradually approached a value almost uniform. It was reported that freshly prepared actomyosin readily dissociates to myosin and actin by the addition of ATP, but becomes difficult to dissociate when stored for a definite period.[7] Comparatively low values for ATP-sensitivity did not seem to be due to this cause; on account of their high ATP-sensitivity of AML from the same sample meats. From the results on ATP-sensitivity of AMS, it was indicated that the constitutional structure of AMS has been changed during aging, and the proportion of myosin in actomyosin becomes larger, namely the chemical nature of AMS approaches that of myosin. The same conclusion will be inferred from the results on salting out previously described.

TABLE II

ATP-SENSITIVITY OF MYOSIN B SOLUTION

Kinds of animal meats	Aging period (Days)	ATP-sensitivity(%) AMS	AML
Rabbit	0	26.5	148.3
	4	132.5	112.5
	8	5.0	97.6
Veal (Holstein Kind)	0	85.5	123.5
	2	26.5	100.9
	4	9.1	100.8
	8	12.1	141.0
	12	4.3	120.1
Veal (Japanese Black Breed of Cattle)	0	34.8	134.8
	2	9.6	137.6
	4	33.8	139.3
	8	1.6	86.5
	12	9.8	98.9
Cow meat	0	23.8	60.5
	2	11.9	131.7
	4	5.6	104.0
	8	3.2	124.0
Horse meat	0	16.1	63.7
	4	52.9	55.8
	8	72.2	76.9
	12	32.9	51.9

ATPase : Changes of optimum pH of myosin B-ATPase during the aging of meats. Some properties of ATPase of AMS and AML have already been reported in a previous paper.[2] The enzymatic activities and the changes of optimum pH values of the two kinds of

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6) Harry L. Seagran, *Food Res.*, **21**, 505 (1956).

7) Szent-Györgyi, Muscular Contraction, 75, 152 (1951), Academic Press Inc., New York.

TABLE III

OPT. pH AND ACTIVITIES OF MYOSIN B ATPASE DURING THE AGING OF MEATS

Kinds of meats	Aging period (Days)	AMS-ATPase		AML-ATPase	
		opt. pH	activity	opt. pH	activity
Rabbit	0	7.3	38.3	7.3	36.4
		10.2	69.7	10.2	90.5
	4	7.3	41.5	7.3	43.1
		10.2	91.3	9.5	50.4
	8	6.2	17.9	6.2	22.0
		10.2	12.3	10.2	29.8
Veal	0	7.1	27.0	6.7	21.4
	2	7.1	13.4	6.7	26.5
	4	6.7	14.7	6.7	23.1
	8	6.7	17.9	6.7	31.5
	12	6.7	16.1	6.2–6.7	12.1
	0	10.2	27.8		
	12	9.0	12.2		
Horse meat	0	6.5	45.2	6.5	23.1
	4	—	—	6.5	25.3
	8	6.5	22.2	6.5	27.4
	12	6.5	13.6	6.5	21.2

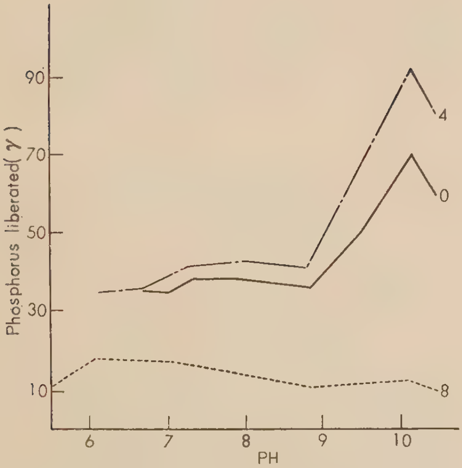

FIG. 3. Opt. pH of ATPase of Rabbit-AMS. Numbers in Fig. show days of aging.

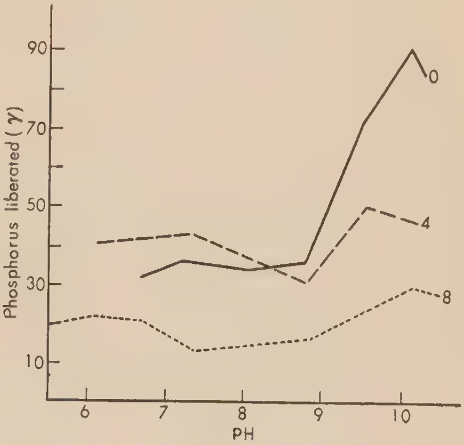

FIG. 4. Opt. pH of ATPase of Rabbit-AML. Numbers in Fig. show days of aging.

ATPases during the aging of meats are presented in Table III and in Figs. 3 and 4.

Table III and Fig. 3 indicate that each ATPase of AMS and AML obtained from rabbit has two pH optima, one near the neutral point (pH 7.3), and the other in the alkaline region (pH 10.2). After 8 days of aging, the optimum pH at 7.3 shifted to the acidic side, i. e., pH 6.2. Also, in myosin B obtained from veal, the optimum pH value shifted to the acidic side in the same manner as in rabbit during the aging of meat, as it can be seen in Table III. Whereas in the case of myosin B from horse meat, no change in the optimum pH was observed during aging process (Table III).

ATPase activity: As shown in Table III, ATPase activity of AMS and AML from rabbit was rather higher at the alkaline region than at the neutral pH, but the difference of enzymatic activities at these two pH values almost diminished after 8 days of aging.

Changes of ATPase activities during aging are described as follows. At the neutral optimum pH, little difference was recognized between the ATPases of AMS and AML. No remarkable changes of the activities were recognized for 4 days, but these activities decreased after 8 days of aging. At the alkaline optimum pH, ATPase activities of AMS showed a maximum on the 4th day of aging, while those of AML decreased with aging.

In Table III, results on ATPase activities of myosin B from veal and horse meat are presented, but the experiments are confronted with activities at the neutral optimum pH value.

In the case of ATPase of AMS from veal, which differed from rabbit, difference was unrecognizable between enzymatic activities at both optimum pH values of 7.3 and 10.2.

Enzymatic activities of myosins from rabbit and veal (the same meat materials as were used to prepare myosin B) at the neutral optimum pH were 38.0 and 29.8 (γ), respectively. Therefore, the ATPase-activity immediately after the slaughter seemed to be in the order: myosin$\geqslant$AMS$\geqslant$AML. On the contrary, in the case of aged meats, enzymatic activity of AML was higher than that of AMS as previously reported.[2]

As shown in Table III, enzymatic activity of veal myosin B was the lowest among the myosin B which were obtained from three kinds of meats (rabbit, veal and horse meat).

SUMMARY

In order to decide whether or not, the chemical properties of myosin B extracted, isolated, and prepared from rabbit, veal, cow, and horse meat have been altered during aging, experiments were carried out on salting out, viscosity, ATP-sensitivity, and the ATPase of myosin B solution.

The results obtained are as follows:

(1) Salting out experiments showed that there is no difference of myosin B to be observed in accordance with the kinds of animals tested. However, in the salting out procedure of AMS and AML solutions, the concentration of ammonium sulfate at the maximum precipitating region was found to be different. The concentration of ammonium sulfate in the salting out of myosin B was altered with the aging of meats; especially, in the case of AMS the maximum precipitating region seemed to approach that of myosin.

(2) Among all the kinds of animal meats tested, intrinsic viscosity of AML was higher than that of AMS, and intrinsic viscosity of myosin B was altered with the aging of meats. Intrinsic viscosity of the myosin B solution seemed to be different according to the kind of meat, i.e., that of myosin B from rabbit being high, while that obtained from cow meat was low.

(3) The ATP-sensitivity of AML was higher than that of AMS. The ATP-sensitivity of AMS became very low as aging proceeded. From this result, it was considered that AMS turns to assume a high ratio of myosin in the constitution of actomyosin, and comes to resemble myosin during aging.

(4) With the aging of meats, the optimum pH for ATPase of myosin B from rabbit and veal meat turned to the acidic side. At the neutral optimum pH, enzymatic activity showed a tendency to decrease during aging. Immediately after slaughter, the activity of ATPase of AMS became higher than that of AML, but the inverse relation was maintained during aging process.

Immediately after slaughter, the optimum pH for enzymatic activity of AMS from rabbit were found to be 7.3 and 10.2, activity at pH 10.2 being higher than that at pH 7.3. However no difference was observed at both

of these pH values in ATPase activity of AMS from veal immediately after slaughter.

Acknowledgements The authors wish to express their sincere thanks to Prof. R. Sasaki, Prof. Y. Sakurai and Prof. M. Kandatsu for their interest in our work, and also to Dr. M. Nakamura for his kind advice.

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Effect of Gamma-Ray upon Food Microorganisms

Studies Concerning Gamma-Ray Resistivity of *Escherichia Coli* (II)

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The author has carried out studies concerning the effects of different conditions on the gamma-ray resistivity of *E. coli*. This report deals with the influence of the heating procedure applied to irradiated samples after irradiation, the gamma-ray resistivities of strains stocked for long periods, and the influences of the addition of heat sterilized cells of the same strain and chemicals on mediums.

INTRODUCTION

In the sterilization of foods with ionizing radiation, destruction of nutrient components, color change, off-flavor and the decomposition of structure of the irradiated foodstuff used, result from extreme irradiation dose. Therefore, the minimum quantity of dose required to destroy the living cell of microorganisms should be investigated in order to minimize these unreasonable changes occurring in foodstuffs. This implies studies concerning the radio-resistivity of various types of microorganisms under different conditions and the comparison of the radio-resistivity of each microorganism.

Studies concerning the effect of post- and pre- treatments on bacterial inactivation have been investigated by many workers. Bayne-Tones has conducted studies concerning the action of X-ray upon microorganisms at varied temperatures and has reported that the lethal action of X-rays, radioactive emanations and ultraviolet light are not affected by temperature, while various physical and chemical agents affect the lethal action of radiation to various degrees within a certain range.1),2),3) Stapleton has conducted studied concerning the survival of an irradiated strain of *E. coli* with X-ray incubated at varied temperatures and has reported that the living cells of the irradiated strain are most survived in the medium, in case of incubation at 18°C.4),5),6)

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1) S. Bayne-Tones, and Van der Lingen, J.S.; The bacterial action of ultraviolet light, *Johns Hopkins Hospital Bulletin*, **34**, 11-16, (1923).

2) F. I., Gates; A study of the bacterial action of ultraviolet light I and II, *J. Gen. Physiol.*, **13**, 231-248 and 249-260, (1929).

3) D. E. Lea., R. B. Haines and C. A. Coulson: The action of radiations on bacteria III, *Roy. Soc. Proc. B.*, **123**, 1-21, (1937).

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5) D. Billen, G. E. Stapleton and A. Hollander: The effect of X-radiation on the respiration of *E. coli*, *J. Bacteriol.*, **65**, 131-135, (1953).

6) B. L. Strehler, G. E. Stapleton and A. Hollander: Post-irradiation release of adenosinetriphosphate from *E. coli* B/r, *Arch. Biochem. and Biophys.*, **43**, 1-10, (1953).

Edwards has conducted studies concerning the survival of *E. coli* irradiated with X-ray in different pH levels of suspension and has reported that no significant difference is to be recognized when samples at either pH 7.0 or pH 5.4 are irradiated.[7] Also, Bayne-Jones reported that a pH below the isoelectric point in a medium containing bacteria greatly enhances the action of ultraviolet light against microorganism.[1] Claus has conducted studies concerning the effect of chemicals in bacterial suspension upon the survival of microorganisms subjected to X-ray irradiation and has reported that the presence of heavy inorganic salts at high concentrations increases the effect of X-ray against the strain of *E. coli*.[8] Stapleton has carried out studies concerning the formation of colonies of irradiated strain of *E. coli* in various mediums and has reported that X- and gamma-irradiated cells markedly decrease the ability to exhibit themselves in both size and the number of colonies on the synthetic medium as compared to complete media.[9] Further, he has also conducted studies concerning the influence of the incubating process in chemical solutions prior to irradiation and states that the strain is apt to survive more longer in case of a dilute solution of succuic acid than in other chemicals.[10]

The author has reported on a study regarding a simplified method for the measurement of gamma-ray resistivity of *E. coli*, giving a comparison of each survival-curve of 11 species of bacteria and also the influence of the addition of chemicals on a medium containing *E. coli* by gamma ray irradiation in a previous report.[11]

In this paper, the effects of different conditions for the survival of *E. coli* irradiated by gamma-ray are reported in sequence of the proceeding report.

EXPERIMENTAL

Normal samples were prepared in the following manner, as explained in detail in the previous report.

The strain of *E. coli* was incubated at 37°C for 24 hours, and the bacteria suspended in a phosphate buffer solution was centrifuged and washed two or three times. After washing, the original suspension was prepared at the concentration of 1 mg/ml (10^8cell/ml) and diluted by the usual serial ten-fold dilution method in a broth solution. The final ten-fold dilution was prepared in a dissolved nutrient agar which was kept at 40°C. Thus, the final bacterial concentration in the nutrient agar was adjusted to 10^2 cell/ml. One-cc of the mixture was pippetted into each small and thin petri-dish, and solidified at room temperature.

All samples were irradiated with cobalt-60 gamma-ray, 30 curie, preserved at the School of Medicine, Keio University, at room temperature within the temperature range of 18°C to 24°C for 2 hours immediately after solidification. After irradiation, both irradiated and non-irradiated samples were instantly placed into an incubator at 37°C, and incubated for 24 hours.

All colonies that developed in each petri-dish were counted by means of a binocular microscopic, with a low magnification. Each individual survival-ratio indicates the percentage obtained from the bacterial counts of irradiated and non-irradiated samples. In this case, control samples were equally treated except in the irradiation procedure.

RESULTS AND DISCUSSION

(1) Experiment 1

The influences of incubating temperature and preserving procedure for 24 hours in an ice-box at 0°C after gamma-ray irradiation on survival ratio of *E. coli* were investigated.

First, one group of the irradiated samples was incubated in an incubator at 37°C for normal strains, and another group was incubated at room temperature, 25°C, for the experiment. The results are diagrammed in Figure 1.

Following this procedure, one group of irradiated samples used for the experiment was placed in an ice-box at 0°C for 24 hours and

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[11] W. Watanabe: Effect of gamma-ray upon food microorganisms -I, This Bulletin, **22**, 2 (68-77), (1958).

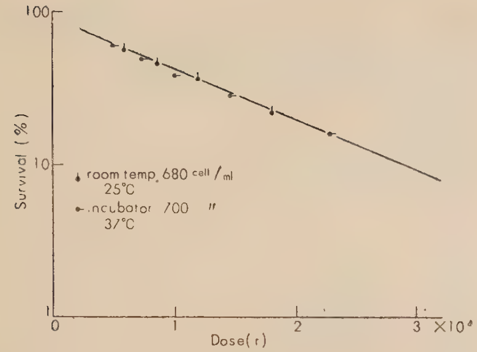

FIG. 1. Influence of Incubating Temperature after Irradiation on Survival Ratio of *Escherichia coli* by γ-Ray Irradiation.

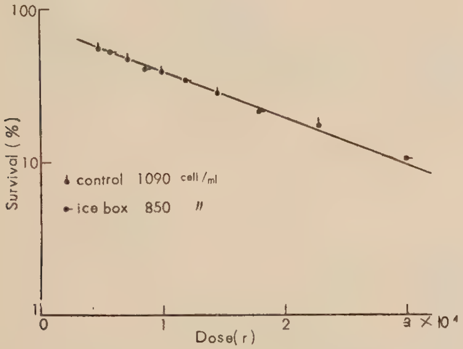

FIG. 2. Influence of Preserving Process in Ice-box for 24 hrs after Irradiation on Survival Ratio of *Escherichia coli* by γ-Ray Irradiation.

subsequently incubated in the same incubator at 37°C for 24 hours. The results are shown in Figure 2.

As it can be seen from both Figures 1 and 2, the survival-curves obtained from samples for normal and experimental strains are identical in both cases. This may indicate that there is no effect of such heating procedure as above-mentioned, no heat reactivation was recognized in these experiments.

(2) Experiment 2

The influence of the stocking period of strains used before irradiation on the survival-ratio of *E. coli* was investigated.

Each strain incubated for 24 hours at 37°C was first stocked at 0°C in an ice-box for a period of 0 to 23 days, and 18°C at room temperature for the second time for 0 to 24 days.

The results in the case of stocking at 0°C are diagrammed in Figure 3. All survival-ratios obtained from the strrins stocked from 0 to 23 days at 0°C were all identical. The gamma-ray resistivity of living cells in the strain may be constant, even if the strain is stocked at 0°C for such a long period. In this case, natural-dead cells were found in each strain stocked for a long period. However, at any rate, no influence of the presence of these natural-dead cells belonging to the same strain was observed on the survival-ratio of living cells of the strain.

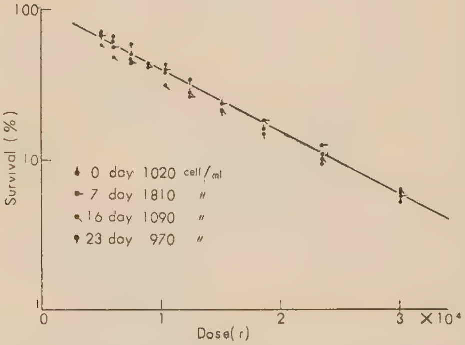

FIG. 3. Influence of Stocking Period in Ice-box (0°C) on Survival Ratio of *Escherichia coli* by γ-Ray Irradiation.

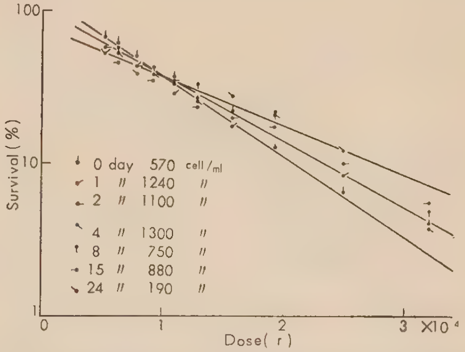

FIG. 4. Influence of Stocking Period at Room Temperature (18°C) on Survival Ratio of *Escherichia coli* by γ-Ray Irradiation.

From the results diagrammed in Figure 4, it is found that in the case of strains stocked at 18°C from 0 to 24 days, the survival-ratio obtained from the strain stocked for a long period is higher than that of the short-period. This may indicate an increasing influence on gamma-ray resistively of living cells in the strain.

Although in this case, natural-dead cells were also found in each strain, it is, however, questionable whether or not the cause of such an influence as above-mentioned may be caused by the co-existence of natural-dead cells at 18°C of the strain.

(3) **Experiment 3**

The influence of dead cells of the strain sterilized by the heating procedure on the survival-ratio of *E. coli* was investigated.

The bacteria suspended in a phosphate buffer solution at the concentration of 10^8 cell/ml was sterilized in water at boiling point for a few minutes, and was then added to each mixture. This suspension contained *E. coli* at the concentration of 10^2 cell per ml. The concentrations of the heat sterilized cells added were 10^7, 10^5 and 10^3 dead-cell per ml.

The results are diagrammed in Figure 5. All survival-ratios obtained from the samples were almost identical, and it can be seen that there may be no influence of the co-existence of heat sterilized cells at the concentration of 10^5-fold on survival of the same living cells caused by gamma-ray irradiation.

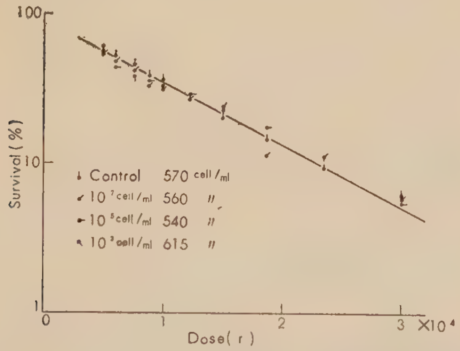

FIG. 5. Influence of Addition of Heat Sterilized Cell of *E. coli* on Survival Ratio of the Same Strain of *Escherichia coli* by γ-Ray Irradiation.

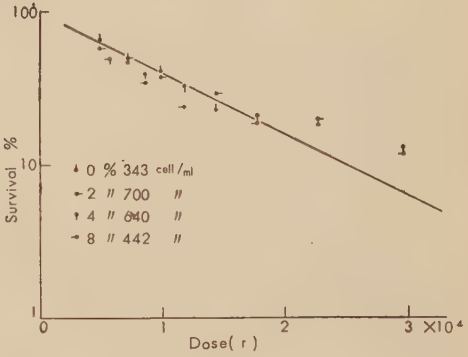

FIG. 6. Influence of Addition of Glucose on Survival Ratio of *Escherichia coli* by γ-Ray Irradiation.

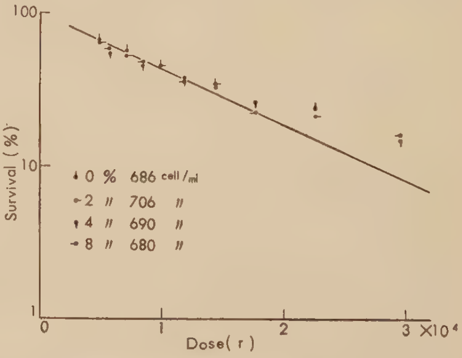

FIG. 7. Influence of Addition of Lactose on Survival Ratio of *Escherichia coli* by γ-Ray Irradiation.

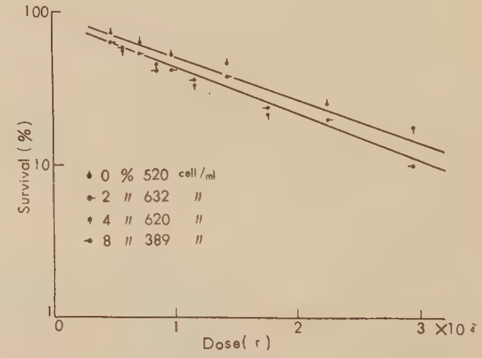

FIG. 8. Influence of Addition of Fructose on Survival Ratio of *Escherichia coli* by γ-Ray Irradiation.

(4) Experiment 4

In a previous report, the addition of sucrose in a medium turned to have no effect upon the survival of *E. coli*. In this experiment, influences of the addition of glucose, lactose and fructose added in each medium upon the survival of *E. coli* by gamma-ray irradiation were investigated.

The concentrations of the chemical substances used were of 2, 4 and 8%. The results are diagrammed in Figures 6, 7 and 8. Figure 6 shows results in the case of glucose, Figure 7 and Figure 8 are results in cases of lactose and fructose.

As it can be seen from the figures, all the survival-curves in each figure were identical, which may indicate that there is no influence of the addition of glucose, lactose or fructose in the medium upod the surviual of *E. coli* by gamma-ray irradiation both during and after irradiation.

(5) Experiment 5

After the above treatment, prior to the experiment of the addition of organic acid, influences of reducing the pH level of the medium were investigated. The mediums used were adjusted by the addition of HCL at pH values of 6.4 and 6.1. The results obtained from mediums of different pH values are diagrammed in Figure 9. The medium of pH 7.0 was used as the sample for normal strains. The survivals obtained from the medium of pH 6.4 slightly differed from those obtained from the medium of pH 7.0. However, the survivals from medium of pH 6.1 were higher than those from the normal medium, which may indicate that gamma-ray resistivity of *E. coli* turn to assume high values in case of the pH value of the medium being abjusted from 7.0 to 6.1.

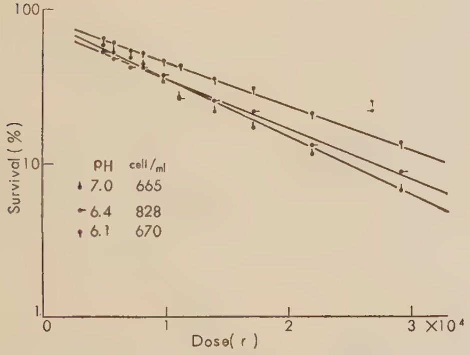

FIG. 9. Influence of Change of pH Value of the Medium on Survival Ratio of *Escherichia coli* by γ-Ray Irradiation.

(6) Experiment 6

Influences of the addition of organic acids to each medium were investigated. Acids examined were tartaric acid, citric acid and succuic acid, and these organic acids were added to each medium in amounts of 0.05,

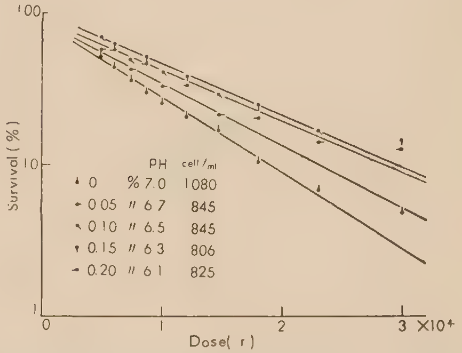

FIG. 10. Influence of Addition of Tartaric Acid on Survival Ratio of *Escherichia coli* by γ-Ray Irradiation.

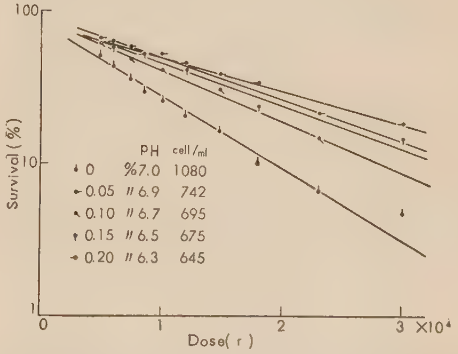

FIG. 11. Influrence of Addition of Citric Acid on Survival Ratio of *Escherichia coli* by γ-Ray Irradiation.

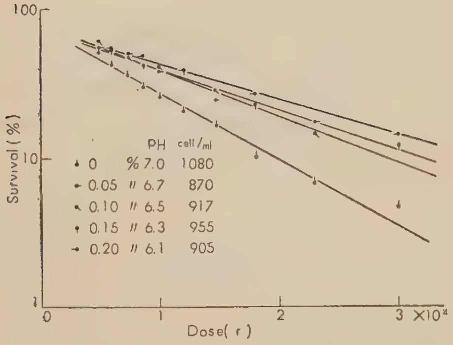

FIG. 12. Influence of Addition of Succuic Acid on Survival Ratio of *Escherichia coli* by γ-Ray Irradiation.

0.10, 0.15 and 0.20 %. In cases of both tartaric acid and succuic acid, the pH value of the mediums added in an amount of 0.20 % was identical, i. e. 6.1. In case of citric acid, the pH was 6.3. The results are diagrammed in Figures 10, 11 and 12, Figure 10 showing tartaric acid, Figure 11 citric acid, and Figure 12 succuic acid. Every survival-curve obtained from each medium containing different acids turned to be the highest at the highest concentration of the chemicals.

When three papers were compared with the paper drawn in the case of HCL addition, every survival-curve obtained from the pH 6.1 mediums added organic acids appeared to be higher than that from HCL medium at the same pH value. Therefore, it may be recognized that the addition of organic acid in a medium containing living cells of *E. coli* at the concentration of 10^2 cell/ml, turns to be more or less protective upon the living cell of *E. coli* irradiated by gamma-ray as a kind of protective agent. In this experiment, the protective effects of three organic acids differed from each other. This fact may indicate that tartaric acid and succuic acid have almost the same effectiveness being more effective than citric acid.

SUMMARY

(1) When samples containing *E. coli* irradiated with gamma-ray were incubated at 37°C or 25°C, no difference on the survival of the strain was observed in both cases and the same survival was also recognized in case of the preserving procedure carried out in an ice-box for 24 hours prior to incubation at 37°C.

(2) Prior to gamma-ray irradiatien, when the strains incubated at 37°C for 24 hours were preserved in an ice-box at 0°C for a period as long as 23 days, no difference on gamma-ray resistivity of the strain was found. However, the survival obtained from the strain stocked at room temperature, 18°C, for a period as long as 24 days was higher than that of the strain stocked for a shorter period.

(3) There was no difference on survival of *E. coli* at the concentration of 10^2 cell/ml caused by gamma-ray irradiation, when the medium also contained heat sterilized cells of the same strain at the concentration of 10^7 cell/ml.

(4) No difference on survival of *E. coli* was caused by gamma-ray irradiation, when the medium contained glucose, lactose or fructose in the amount of 8 %.

(5) The survival obtained from the medium at the pH value of 6.4 adjusted with HCL was almost identical with that of the pH 7.0 medium. However, when the pH value of the medium decreased to 6.1, survival obtained from the medium was higher than that in the medium of pH 7.0 or 6.4.

(6) When tartaric acid, citric acid or succuic acid was added to each medium, the survival-ratios obtained from the medium containing one kind of the chemicals were higher than that of the normal medium containing no organic acid. In these cases, tendencies of such protective effects as previously mentioned were more distinct than in the case of the medium containing HCL at the same pH value, 6.1.

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A Study of Ascorbic Acid Synthesis with Green Peas Seedling

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Concerning the metabolic pathways of ascorbic acid synthesis, an attempt was made to prepare a soluble enzyme preparation from green peas seedlings, since Mapson et al. obtained mitochondria from cress seedling which was able to synthesize L-ascorbic acid from sugar derivatives, and also a soluble enzyme preparation which was used with the mitochondria, the reaction mechanisms being informed. By the application of this preparation, though it was crude, the reaction route, which seems to be operated in rats and cress seedling and is not catalized in an appreciable rate with Mapson's preparation, was thus studied; namely the reaction was considered to pass via 2-keto-L-gulonic acid.

INTRODUCTION

In the view of a similar configuration, one of the hexose sugars has long been considered to be a precursor to L-ascorbic acid synthesis in vivo. A number of works suggested the relation to the synthesis of ascorbic acid and hexose sugars in plants and animals. However, none of them indicated the direct pathway. Recently, King et al. in their experiments feeding the labeled glucose to rats have shown that the intact chain of glucose could serve as a precursor for L-ascorbic acid.[1),2),3)] Further studies with rats revealed the reaction to pass from D-glucuronolactone to L-ascorbic acid via L-gulonolactone.[4),5)] Loewus et al. also observed that labeled D-glucose was converted to L-ascorbic acid without inversion of the carbon chain, when the labeled sugars were injected into ripening strawberries.[6)]

Independently, Mapson et al. observed that D-glyceric acid methyl ester did not increase ascorbic acid formation, though it appeared to be in parallel with ascorbic acid synthesis during cress seedling.[7)] Extensive studies, concerning the metabolism and a precursor of ascorbic acid with various hexose sugar

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[1)] S. S. Jackel, E. H. Mosbach, J. J. Burns and C. G. King, *J. Biol. Chem.*, **186**, 569 (1950).

[2)] H. H. Horowitz, A. P. Doerohuk and C. G. King, *J. Biol. Chem.*, **199**, 193 (1952).

[3)] H. H. Horowitz and C. G. King, *J. Biol. Chem.*, **200**, 125 (1953).

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[5)] J. Burns and C. Evance, *J. Biol. Chem.*, **223**, 897 (1956).

[6)] F.A. Loewus, R. Jang and C. G. Seegmiller, *J. Biol. Chem.*, **222**, 649 (1956).

[7)] F. A. Isherwood, Y. T. Chen and L. W. Mapson, *Bochem. J.*, **56**, 15 (1954).

derivatives, were carried out by these workers, using cress seedling and rats. Among various sugar derivatives, only L-gulono, L-galactono-, and D-glucurono-γ-lactones and D-galacturonic acid methyl ester were shown to be effective for ascorbic acid synthesis.[8),9)] The series of reactions thus postulated by them were as follows:

D-glucose→D-glucuronic acid→L-gulonic acid
→L-ascorbic acid (1)
D-galactose→D-galacturonic acid
→L-galactonic acid→L-ascorbic acid (2)

With the mitochondria and a soluble enzyme of cress seedling, the enzymic conversion in the above reactions was further studied, the mechanism of reaction being informed.[10)] As reaction route (1) was catalyzed in a negligible extent with their preparation, the study was limited within the reaction of route (2).

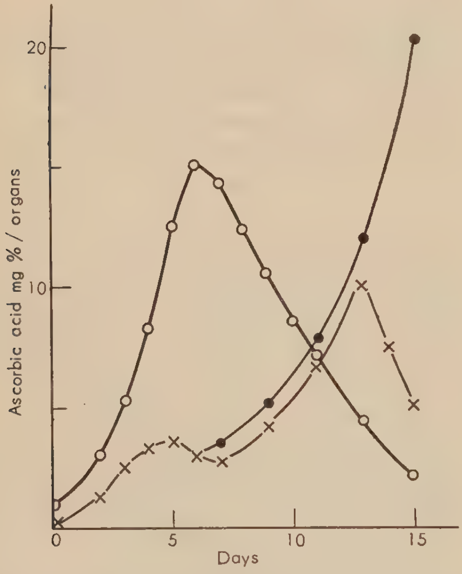

FIG. 1. Ascorbic Acid Contents was Measured in Different Organs During Seedling.

○——○: Cotyledon, ●——●: Embryo, and ×——×: Root.

For the purpose to study the reaction mechanisms concerning with above routes, the author made an attempt to prepare the soluble enzyme fraction from green peas seedling and studied several properties of this fraction. In the preparation, the activity of crude enzyme was however completely lost during dialysis and the activity was not recovered by the addition of the dialyzed solution. Thus, the mechanisms and properties of overall reactions with crude enzyme solution will be presented in this paper.

EXPERIMENTAL

Sample. In general, ascorbic acid increases in a large quantity during peas seedling. With green peas, the increment of ascorbic acid contents according to the date of seedling was determined (Fig. 1). When seedling was homogenized and extracted with phosphate buffer, ascorbic acid disappeared. By incubation of this extracted solution at 30°, ascorbic acid gradually reappeared. After certain periods of seedling, cotyledones, embryos and roots of green peas seedlings were separated, and each fraction was homogenized using Potter's homogenizer with 0.1 M phosphate buffer of pH 6.0. The ascorbic acid synthesized after 24 hours' incubation of the solutions was determined and the

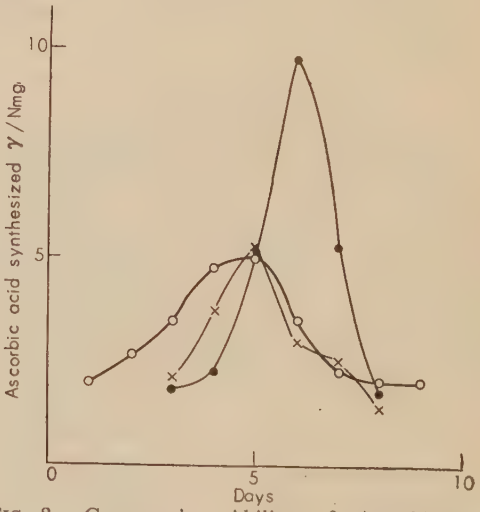

FIG. 2. Comparative Ability of Ascorbic Acid Synthesis with Different Organs of Green Peas Seedling. Activity was Shown According to N mg.

○——○: Total peas, ●——●: embryo, and ×——× root.

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8) F. A. Isherwood, Y. T. Chen and L. W. Mapson, *Biochem. J.*, **56**, 1 (1954).

9) F. A. Isherwood, Y. T. Chen and L. W. Mapson, *Nature, Lond.*, **171**, 348 (1953).

10) L. W. Mapson, F. A. Isherwood and Y. T. Chen, *Biochem. J.*, **56**, 21 (1954).

results are shown in Fig. 2. Nitrogen was determined by the micro Kjeldahl method.

Preparation. In order to prevent bacterial infection, green peas were immersed in 1% mercuric dichloride solution for one min., rinsed in running water over night and washed several times with sterilized water. The peas were thus germinated on the sand previously treated with 10% HCl and washed thoroughly with sterilized water. Seedling was performed at room temperature in the dark. Since the six-day old embryo was the most active, this was used as an enzyme material. After six days, the peas were collected and washed with distilled water. The embryos were separated from other organs and homogenized with an approximately equal volume of 0.1 M pohsphate buffer of pH 6.0 at 0–5°, using a Werling blender. The homogenate was centrifuged at 12,000 r. p. m. for 20 min. at 0°. The supernatant obtained was used as crude enzyme solution.

Reaction of crude enzyme solution. Two ml. of crude enzyme solution prepared by the above procedure, and 0.5 ml. substrate solution were pippeted in a sterilized test tube, and the total volume was made up to 5.0 ml. with distilled water. The test tube was cotton stoppered and incubated in a thermostat at 30°. After an appropriate period, 2 ml. of 5% metaphosphate solution was added to the tube, and the ascorbic acid synthesized was determined by Fujita's method,[11] after being titrated with 2,6-dichlorophenolindophenol.

Paper chromatography. Identification of 2, 6-dichlorophenolindophenol reducing substance was tested by paper chromatography according to the method of Chen et al.[12] A filter paper (Toyo Roshi No. 2 of 30×30 cm) was pregnated with 2% of oxalic acid and dried in air. The sample was developed by two-dimensional paper chromatography, treating the solvents of the upper layer of *n*-butanol : aqueous oxalic acid saturated solution=6 : 4 (20°C) and the lower layer of phenol : aqueous oxalic acid saturated solution=5 : 1 (20°C). After the development at 18°, the paper was dried in air ands preaded with 2,6-dichlorophenolindophenol solution (0.04 g. in 1 l. of 0.25 M Na_2HPO_4). The reducing substance developed a white spot, while the background was colored pink.

The sample was however too dilute to perform paper chromatography directly, condensation being required. After the incubation for 24 hours, the reaction solution was spotted at 5 cm. from the bottom of a 30×30 cm. of oxalic acid pregnated filtter paper and the paper was developed with the solvent of *n*-butanol and aqueous saturated oxalic acid solution. Guided by pure ascorbic acid, the fraction was cut and extracted with glass distilled water. With 10% calcium acetate, the extract was adjusted to pH 2 and then centrifuged. The supernatant was dried over P_2O_5 in a vaccum desicator. The concentrated solution was then chromatographed. The 2, 6-dichloroindophenol reducing substance was identified as L-ascorbic acid, showing its R_F value as 0.28 and 0.41. By direct condensation of reaction without preliminary treatment of paper chromatography, only one spot was shown which was identified as L-ascorbic acid.

Substrates. D-Gluconic acid was synthesized by Kiliani's method,[13] oxidizing D-glucose with brom and D-galacturonic acid was prepared by hydrolyzing pectin with H_2SO_4 according to the procedure of Ishikawa.[14] Diacetone-2-keto-L-gulonic and 2-keto-L-gulonic acids were obtained from Takeda Pharmaceutical Co. by the courtesy of Dr. Sato. D-Glucurono-γ-lactone was a commercial product obtained from Chugai Pharmaceutical Co. and was used after recrystallization.

RESULTS

Fractionation. The supernatant (F_1) and the precipitants (F_2) obtained after centrifuging at 3,000 r.p.m. for 5 min. and the supernatant (F_3) by second centrifugation of F_1 at 12,000 r.p.m. for 20 min. at 0° were incubated in the above system and ascorbic acid synthesized was estimated. The results are shown in Table I. Fraction 3 which showed the highest activity was thus further studied in regard to

TABLE I

Fraction	Ascorbic acid γ/ml
1	4.9
2	0.8
3	5.0

Fraction 1 : Supernatant of 50 g embryo extracted solution with equal amount of 0.1M phosphate buffer of pH 6.0, after centrifuging at 3,000 r.p.m. for 5 min.

Fraction 2 : After centrifuging Fraction 1. at 12,000 r.p.m. for 5 min., the precipitates being suspended with 10 ml. of 0.05M phosphate buffer of pH 6.0.

Fraction 3 : The supernatant by the preparation of Fraction 2.

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[11] A. Fujita, Determination of Vitamins, p. 157 (1946) Seibundo, Tokyo.

[12] Y. T. Chen, F. A. Isherwood and L. W. Mapson, *Biochem. J.*, **55**, 821 (1953).

[13] H. Kiliani und S. Kleemann, *B.*, **17**, 1296 (1884).

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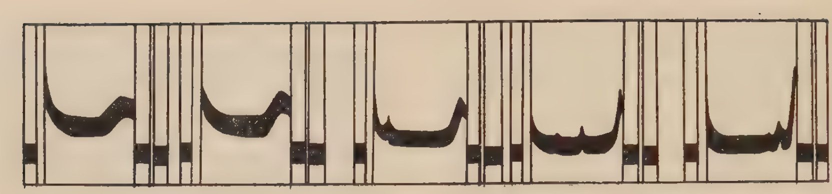


FIG. 3. Ultracentrifuge Diagram of Pea Extract.

A, B, C, D and E show the diagram after constant speed of 42,000 r.p.m. for 8, 16, 24, 32 and 40 min. at 10°.

its physical properties by ultracentrifugation, using a Spinco Model E. The fraction was centrifuged at 42,000 r.p.m. for 16 min., the results being shown in Fig. 3. Three fractions were observed of which sedimentation constants (s_t) were 65, 41 and, 2.2×10^{-13} respectively. For the activity test, F_1 was thus centrifuged at 42,000 r.p.m., using separation tubes of 1.5×6 cm. After 120 min., the solution was gently pippeted 2.5 ml., each from the upper fraction. Each fraction was incubated with D-glucuronolactone and ascorbic acid synthesized was estimated (Table II). The third fraction of $s_t = 2.2 \times 10^{-13}$ had the highest activity, thus, the supernatant after the centrifugation at 15,000 r.p.m. for 20 min. was used for the following experiments.

TABLE II
ACTIVITY OF FRACTIONS AFTER ULTRACENTRIFUGE

Fraction	Ascorbic acid γ/N mg
1	1.78
2	1.50
3	1.43
4	0.63

Centrifuge was followed for 120 min., at 42,000 r.p.m. at 0°C. Each fraction of 2 ml. was incubated with 0.5 ml. of 1% glucuronolactone for 24 hrs. at 30°.

Fraction 1: 2.5 ml. from the upper layer of a centrifuging tube, 2: 2.5 ml. of the second layer, 3: 2.5 ml. of the third layer, and 4: the final fraction to the bottom.

Dialysis of the crude enzyme solution. During dialysis of the crude enzyme solution prepared by the above method, the activity was completely lost. The solution was dialyzed against distilled water, 10%, 1% and 0.1% of NaCl aqueous solutions, and phosphate buffers of 0.1M and 0.05 M of both pH 6.0 and 7.8 at 0° for 24 hours. The dialyzed solutions lost the activity in every case. To recover the activity, boiled crude enzyme solution, dialyzed outside solution against distilled water and boiled yeast extract solution were added to the reaction system respectively. The activity was however not recovered by any of them. It seemed probable that one or more of the enzymes concerned with the overall reaction would be inactivated during the dialysis and the inactivation would not be due to the loss of coenzymes. Because of the difficulty in purification of this crude enzyme solution, the following experiments were performed with the crude solution, not being purified further.

Optimum pH and temperature. Using D-glucurono-γ-lactone as the substrate, the crude enzyme solution was incubated at 30° for 24 hours with phosphate buffers of 0.05 M by varing their pH from 5.5 to 7.2. The pH of solutions was measured by a glass electrode pH meter. The optimum pH was shown to be at pH 5.8–6.2 (Fig. 4). The optimum temperature appeared to be in the range of

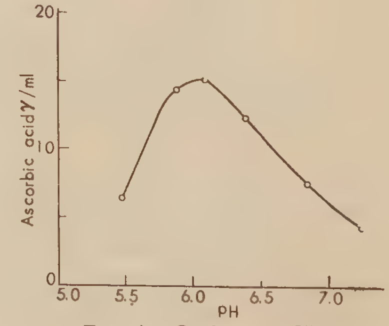


FIG. 4. Optimum pH.

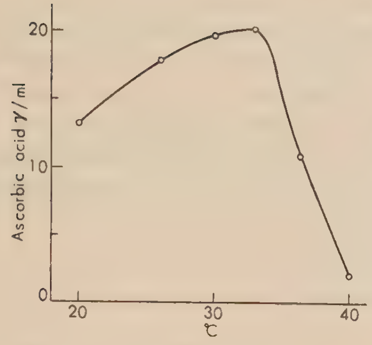


FIG. 5. Optimum Temperature.

30–33°, the solution being incubated with phosphate buffer of pH 6.0 and 0.05 M. By further increment of temperature rapidly reduced activity (Fig. 5).

Progress of the reaction. In the system described above, the enzyme solution was incubated with D-glucurono-γ-lactone, at 30° for periods ranging from 8 to 30 hours. As shown in Fig. 6, the reaction was not in the first-order, but seemed to require some lag phase. If the reaction takes place from glucuronic acid to ascorbic acid through L-gulono-γ-lactone, the results indicated multiple reaction systems. Since the crude enzyme solution was not dialyzed, it contained precursors for ascorbic acid. The overall reactions seemed to be limited at some step even in the presence of dializable substances, including precursors.

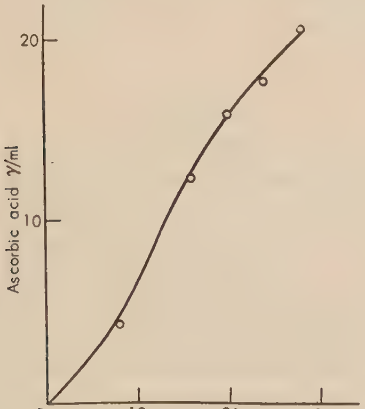


FIG. 6. Reaction proceeding by times.

TABLE III EFFECT OF SUBSTRATES

Substrate	Ascorbic acid γ/ml	
D-Glucurono-γ-lactone	10.25	17.04
D-Galacturonic acid	10.30	17.15
D-Gluconic acid	7.32	11.92
D-Galactose	4.60	11.60
D-Glucose	5.90	11.15
Diacetone-2-keto-L-gulonic acid	—	46.00
2-Keto-L-gulonic acid methyl ester	—	16.00
Control	6.95	13.56

Enzyme solution was incubated with 1% substrates for 24 hrs. at 30°C.

Substrates. The effects of the substrates are shown in Table III. D-Glucurono-γ-lactone and D-galacturonic acid were higher, and D-glucose and D-galactose were lower than the control, while L-gluconic acid was a little effective. Diacetone-2-keto-L-gulonic acid was the most effective substrate, being possibly shown to be a direct precursor with this preparation. 2-Keto-L-gulonic acid methylester was not effective, though it was slightly effective than the control. As a precursor, the free carboxyl group was thus considered to be important. The reaction was probably considered to proceed through 2-keto-L-gulonic acid. For the lower synthetic results with D-glucose and D-galactose, it is considered that these substances are metabolized not only in ascorbic acid synthesis, but also in other reactions which utilize ascorbic acid itself, or require some substances which are necessary for ascorbic acid synthesis.

Inhibitors. The tested reagents were KCN, NaF, 2,4-dinitrophenol, iodoacetate and mercuric chloride of which the results are shown in Table IV. KCN and NaF inhibited the reaction at the concentration of 10^{-3} M, but only slightly at 10^{-4} M. No inhibition by 2,4-dinitrophenol was observed, consideration being taken that the reaction may not be related with oxidative phosphorylation, though the enzyme preparation excluded particurates. The group of SH-inhibiting reagents such as iodoacetate and heavy metals inhibited the reaction completely, though the heavy metals themselves are oxidizing reagents for ascorbic acid.

TABLE IV EFFECT OF INHIBITORS

Inhibitor	Conc.	Ascorbic acid γ/ml
KCN	10^{-3} (M)	7.8
〃	10^{-4}	10.3
NaF	10^{-3}	8.4
〃	10^{-4}	9.5
2, 4-Dinitrophenol	10^{-4}	10.5
Iodoacetate	10^{-4}	0
$CuCl_2$	10^{-4}	0
$HgCl_2$	10^{-4}	0
Control	—	10.5

Heat stability. After heating the enzyme solution at 50°, for 30 sec. and 1 min., and at 100° for 30 sec., the solution was incubated with D-glucurono-γ-lactone for 24 hours. The ascorbic acid synthesized was estimated respectively (Table V). At 50° the enzyme was relatively stable, while at 100° activity rapidly disappeared.

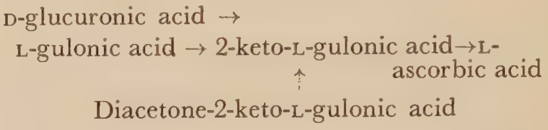
TABLE V HEAT STABILITY

Temp. (°C)	Time (min.)	Ascorbic acid γ/ml
50	0.5	16.9
50	1.0	14.2
50	2.0	13.6
100	0.5	0
Control	—	17.0

DISCUSSION

With crude enzyme solution, L-ascorbic acid could be synthesized from D-galacturonic acid, D-glucurono-γ-lactone, and diacetone-2-keto-L-gulonic acid which had the highest effect. This enzyme preparation was thus considered to be capable of catalyzing the reaction from D-glucurono-γ-lactone to L-ascorbic acid through L-gulonic acid, while Mapson's soluble enzyme preparation appeared to be highly effective to the other reaction, that is the reaction from D-galacturonic acid through L-galactonic acid to L-ascorbic acid.

By Mapson and Breslow,[15] an enzyme which specifically catalyzes the oxidative reaction from L-galactono-γ-lactone to L-ascorbic acid was partially purified from an acetone powder extract of cauliflower mitochondria, and the reaction route (2) has been confirmed by soluble enzyme system. However, the preparation used in this experiment was considered to catalyze as follows:



D-glucuronic acid →
L-gulonic acid → 2-keto-L-gulonic acid→L-ascorbic acid
↑
Diacetone-2-keto-L-gulonic acid

The preparation excluded any particles and dinitrophenol showed no effect to ascorbic acid synthesis, thus, the oxidative phosphorylation coupling reaction seems to be unrelated in overall reactions.

Hence, 2-keto-L-gulonic acid methyl ester was not effective, free carboxyl group is thus considered to be required.

By dialysis, the activity was completely lost irreversibly and activity was not generated, even with the addition of boiled yeast extract and boiled solution of pea extract. Since the dialyzed solution was not tested with 2-keto-L-gulonic acid, it is not to be discussed here whether the loss of activity is due to coenzymes or disintegration of enzyme systems. This problem will be further studied, using a substrate such as 2-keto-L-gulonic acid with this preparation, limiting the reaction within a simple step.

SUMMARY

The soluble enzyme preparation which can synthesize L-ascorbic acid from D-glucuronic acid, D-galacturonic acid and diacetone-2-keto-L-gulonic acid was obtained from green peas seedling. During dialysis of this preparation, a loss of activity was however caused and it was irreversibly generated by any addition of the dialyzed solution, boiled enzyme solution and boiled yeast extract. The experiments were carried out without dialysis and further purification. The active fraction showed its

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[15] L. W. Mapson and E. Breslow, *Biochem. J.*, **65**, 30 p (1957).

sedimentation constant as $s_t=2.2\times10^{-13}$, indicating a relatively lower molecular weight of protein.

The optimum pH and temperature of the soluble enzyme was pH 5.8–6.2 and 33° respectively. The enzyme was relatively stable at 50° for a short period of heating, but was rapidly inactivated at 100°. Inhibition was observed with iodoacetate, $CuCl_2$ and $HgCl_2$; KCN and also NaF slightly inhibited at 10^{-3} M, while 2,4-dinitrophenol showed no inhibition to this preparation.

Acknowledgement. The author is grateful to Prof. Y. Oshima for his valuable advice, and to Mr. and Mrs. Hayashi for their generous assistance in performing ultracentrifuge analysis.

A Novel Route of Synthesis to Chrysanthemum-dicarboxylic Acid

Sir:

Chrysanthemum-dicarboxylic acid can exist theoretically in four geometrically isomeric forms. Two of these isomers, i. e. *trans*, *trans*-, and *cis*, *trans*-acids with *trans*-side chain, have already been synthesized[1] and their geometrical configurations have also been established by experimental evidence provided by the authors.[2]

Through the authors' route of synthesis previously reported, however, the remaining two isomers cannot be obtained because of the *trans*-configuration of the parent $\alpha\delta$-dimethylsorbic acid ester employed as the starting material. Then, the authors' attension was directed to an alternative scheme which would promise the formation of chrysanthemum-dicarboxylic acids with *cis*-side chain.

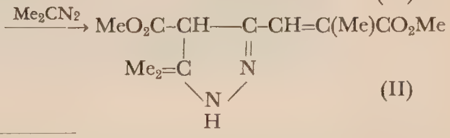
Concerning the addition mechanism of aliphatic diazo-compounds to the conjugated diene systems substituted by polar groups, discussions based on the existing data have been made and some theoretical conceptions have been developed by the authors.[3]

According to this, it seemed likely that the $\gamma\delta$-addition of dimethyldiazomethane to α-methylmuconic acid ester, which would be necessitated for the successful synthesis of chrysanthemumdicarboxylic acid, would predominate. Nevertheless, in the sole existing case of analogous type of reaction, Guha and Sankaran[4] observed the terminal (1, 4-) addition of dimethyldiazomethane and diazomethane to ethyl *trans*, *trans*-muconate, yielding 2, 2-dimethyl-*cyclo*pent-4-ene-1, 3-dicarboxylate and *cyclo*pent-ene-1, 3-dicarboxylate respectively. Here, the situation differs in that *trans*, *trans*-muconate carries no α-alkyl substituent and has a molecular symmetry in contrast to α-methylmuconic acid, in which the electron-releasing α-methyl substituent reduces the polarization of the $\alpha\beta$-double bond induced by α-carboxyl group as compared with that of $\gamma\delta$-double bond and lacks molecular symmetry. It was for this purpose that the authors have prepared the hitherto undescribed isomers of α-methylmuconic acid and established their geometrical configurations in the preceding paper.[5] Again, the successful synthesis of chrysanthemum-dicarboxylic acid would necessitate the $\gamma\delta$-addition of dimethyldiazomethane to α-methylmuconic acid ester and, if it should be the case, this method of synthesis would be advantageous in that the geometrical configuration of the parent isomeric α-methylmuconate represents that of the adduct, i. e., chrysanthemum-dicarboxylic acid.

Dimethyldiazomethane was added to methyl α-methyl-*trans*,*trans*-muconate in chilled (−18°) xylene solution for 18 hours.

After removal of xylene, a crystalline pyrazoline ester, m.p. 144–5° (*Anal.* Found C, 56.59, H, 7.01; Calcd. for $C_{12}H_{18}O_4N_2$ C, 56.68, H, 7.14) was isolated pure as an intermediate and its structure was confirmed as Δ^2-pyrazoline (II) by infra-red (N–H 3270 cm^{-1}, carboxyl C=O 1712 cm^{-1}, C=N 1675 cm^{-1}) and ultra-violet spectra ($\lambda_{max.}$ 213 mμ, ε 13,200; 299 mμ, ε 9,600).

$MeO_2C{-}CH{=}CH{-}CH{=}C(Me)CO_2Me$ (I)



(II)

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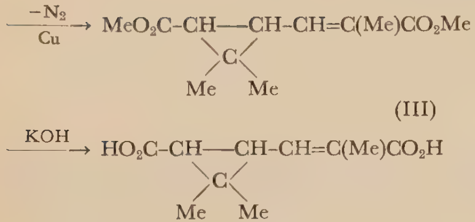
[1] Y. Inouye, Y. Takeshita and M. Ohno, This Bulletin, **19**, 193 (1955).

[2] Y. Inouye, T. Sugita and M. Ohno, This Bulletin, **21**, 5, 222 (1957).

[3] Y. Inouye and M. Ohno, *Bull. Inst. Chem. Res. Kyoto Univ.*, **33**, 237 (1955).

[4] P. C. Guha and D. K. Sankaran, *Ber.*, **70**, 2109 (1937).

[5] T. Sugita, Y. Inouye and M. Ohno, This Bulletin, **22**, 162 (1958)


$-N_2$
Cu
MeO_2C-CH—CH-CH=C(Me)CO_2Me
C
Me Me
KOH
HO_2C-CH—CH-CH=C(Me)CO_2H
C
Me Me

(III) (IV)

The thermal decomposition of the pyrazoline (II) in the presence of copper catalyst took place at an elevated temperature (170–180°) under vigorous evolution of nitrogen; and subsequent distillation *in vacuo* of the residue gave, as was expected, methyl *trans*, *trans*-chrysanthemum-dicarboxylate, m. p. 78–9° (III).

This crystalline ester as well as the corresponding free acid, m.p. 206–8° (IV), which was obtained by alkaline hydrolysis of the ester, were completely identical with the respective authentic specimen previously obtained through a different route by the authors.(1) This was shown by the mixed melting point comparison as well as by the complete identity of infra-red and ultra-violet absorption spectra. The formation of *trans*, *trans*-chrysanthemumdicarboxylic acid from α-methyl-*trans*, *trants*-muconic acid by the addition of dimethyldiazomethane provides another evidence for the geometrical configuration concluded in the previous papers.

The addition of dimethyldiazomethane to methyl α-methyl-*cis*, *trans*-muconate gave a pyrazoline, m. p. 99–100° (*Anal.* Found C, 56.65, H, 7.27; Calcd. for $C_{12}H_{18}O_4N_2$ C, 56.68, H, 7.14); IR, N–H 3230 cm^{-1}, carboxyl C=O 1715 cm^{-1}, C=N 1672 cm^{-1}; UV, $\lambda_{max.}$ 213 mμ (end absorption) ε 10,700, 299 mμ ε 10,200. By the same procedure, methyl α-methyl-*cis*, *cis*-muconate gave three pyrazolines: (i) m.p. 94–5° (*Anal.* Found C, 56.56, H, 7.34; Calcd. for $C_{12}H_{18}O_4N_2$ C, 56.68, H, 7.14); IR, N–H 3260 cm^{-1}, carboxyl C=O 1718 cm^{-1}, C=N 1678cm^{-1}; UV, $\lambda_{max.}$ 213 mμ (end absorption) ε 8,500, 299 mμ ε 8,100. (ii) m.p. 151° (*Anal.* Found C, 55.65, H. 7.74, N, 17.06; Cald. for $C_{15}H_{24}O_4N_4$ C, 55.54, H, 7.46, N, 17.27); UV, $\lambda_{max.}$ 214 mμ (end absorption) ε 3,580, and (iii) m. p. 99–100°.

Both analytical and spectral data showed the pyrazoline (ii) to be a bicyclic pyrazoline which resulted from 1,4-addition of one molecule of dimethyldiazomethane and subsequent addition of another molecule of dimethyldiazomethane to the double bond created by the rearrangement. The third pyrazoline (iii) was identical with that obtained from methyl α-methyl-*cis*, *trans*-muconate and is assumed to have resulted from isomerization during addition.

Experiments are now in progress to establish definitely the geometrical configuration of isomeric chrysanthemum-dicarboxylic acids with *cis*-side chain and acyclic by-products obtained by the thermal decomposition of the above-mentioned pyrazolines. Details will be described elsewhere in the near future.

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[Bull. Agr. Chem. Soc. Japan, Vol. 22, No. 4, p. 271~272, 1958]

Isolation of D-lyxuronic acid as an oxidative metabolite from glucose by *Acetobacter melanogenum*

Sir:

It has been reported by the authors[1] that the growing *A. melanogenum* produces an unknown penturonic acid-like substance, following the production of 2, 5-diketogluconic acid, in a medium consisting of 10% D-glucose, 0.8% yeast extract, and 2% calcium carbonate.

Considerable amount of this unknown acid was isolated from the thirty day-culture of *A. melanogenum* under static condition. After decolorization with lead sulfide and active carbon, the broth was concentrated to syrup, and added ethanol up to 50%. From the supernatant, the calcium salt was precipitated at 80% of ethanol. Then the aqueous solution was fractionated to acidic and non-acidic parts by passing through an Amberlite IR-4B ($CO_3^=$) resine column. The non-acidic fraction contained three sugars, which revealed spots of R_F 0.55, 0.40, and 0.25 on paper partition chromatogram (*tert*-BuOH : HCOOH : H_2O = 4 : 1 : 1.5). These spots were developed respectively to pink-red, brown, and brown by aniline-oxalate, and to blue, green, and blue by phloroglucin-HCl as the spray reagents. In the acidic fraction, after passing through an Amberlite IR-120 (H^+) column, an acid was detected, which had a spot of R_F 0.40, developing to yellow by brom phenol blue, to pink-red by aniline-oxalate, and to green by phloroglucin-HCl reagents. Crystalline calcium salt of this acid was obtained in acetone solution for a few months, but the amount was not plenty enough. The lyophilized calcium salt, yielded about 5 g., was analized as follows.

Crystals of Ca-D-lyxuronite furanoside.

This calcium salt showed the typical pentose reactions with orcin- and phloroglucin-HCl

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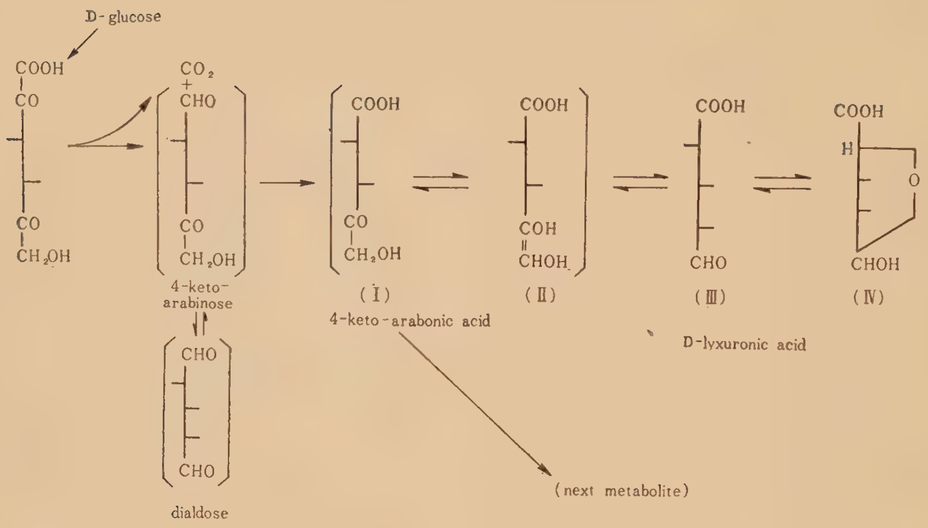
[1] K. Kondô, M. Ameyama and T. Yamaguchi, *Nippon Nōgei Kagaku Kaishi*, **30**, 419 (1956): *C. A.*, **51**, 18104 c.



and aniline-acetic acid paper tests. Moreover, it gave the typical uronic acid reactions with naphthoresorcin-HCl and carbazol-H_2SO_4 tests. It reduced strongly ammoniacal silver nitrate and Benedict's reagents, and also produced crystals with semicarbazine and phenylhydrazine. An infra-red spectrum of the solid calcium salt showed the absorptions (803, 870, and 925 cm^{-1}) of typical furanose ring, though typical carbonyl absorption (ca. 1700 cm^{-1}) has not been observed. $[\alpha]^{20}_{D}$ was $-23°$ and changed to $-53°$ after thirty minutes. *Anal.* Found: C, 27.45; H, 5.09; Ca, 10.79; water of crystallization 15.25. Calcd. for $C_5H_7O_6$. Ca/2·$2H_2O$: C, 27.40; H, 5.06; Ca, 9.14; water of crystallization 16.44. Its aqueous solution consumed three moles periodate per mole at 0°C, and glyoxylic and formic acids were detected as the oxidation products. The same products were obtained by the action of hydrogen peroxide in alkali. By bromine oxidation, it was oxidized to D-arabo-trihydroxyglutaric acid[2,3], which was crystalized after removal of cation and was found to melt at 127° to 129° and its $[\alpha]^{20}_{D}$ was ca. $+23°$.

These results establish that the substance is calcium D-lyxofuranose-uronate.

Authors have assumed that D-lyxuronic acid is produced from D-glucose via 2,5-diketogluconic acid and probably via "4-ketoarabinose" and "4-ketoarabonic acid". Two spots showing R_F 0.40 and 0.55 in the non-acidic fraction mentioned above have been presumed as 4-ketoarabinose, which is produced by decarboxylation from carboxyl group of diketogluconic acid, and its isomer "dialdose."

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Received May 29, 1958

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2) Tollens and Rorive, *Ber.*, **40**, 2435 (1907).
3) E. v. Lippmann, *Ber.*, **26**, 3059 (1893); **32**, 1213 (1899).

[Bull. Agr. Chem. Soc. Japan, Vol. 22, No. 4 p. 273~274, 1958]

Microbiological Hydroxylation of Reichstein's Compound S (17α-hydroxydesoxycorticosterone) by *Sclerotinia* sp.; 2β-Hydroxylation

Sir:

For the last few years extensive studies on microbiological hydroxylations have shown that several numbers of the position of the steroid nucleus are hydroxylated. Of these hydroxylations by microorganisms, 2β-hydroxylations of Reichstein's Comp. S have just recently been discovered involving only two kinds of microorganisms, i.e. *Streptomyces*1) sp. and *Rhizoctonia*2) sp.

We have found that, strains other than the above mentioned, *Sclerotinia* sp. hydroxylate Reichitein's Comp. S at C-2 position.

These microorganisms were also capable of hydroxylation at C-11 position of the steroid. Here upon, we wish to report on the preparations of 2β-, 11α- and 11β-hydroxylated steroids by *Sclerotinia* sp.

In our experience reported here, *Sclerotinia libertiana* was incubated in 2 l. of potato-decoct media containing glucose.

After incubation at 26° for a period of 48 to 72 hours on a rotary shaker, 1 g. of Reichstein's Comp. S in a methanol solution was added and incubation was carried on for an additional 72 hours. At the end of this period, the oxygenated products were extracted with ethylacetate and extracts were concentrated to dryness in vacuo.

Paper chromatography of this crystalline residue showed one major oxgenated product and the two other products. No unreacted Reichstein's Comp. S was detected on this paper.

To separate them, this residue was dissolved in ethylenedichloride and chromatographed on Florisil. Ethylenedichloride-acetone solvent mixtures were used as developers.

The first-fractions obtained from the column contained the 2β-hydroxylated product, the middle fractions contained the 11β-hydroxylated product and the last one contained the 11α-hydroxylated product.

The product obtained by recrystallization of the first fractions, viz the major product by this microorganism was, 2β, 17α, 21-Trihydroxy-4-pregnene-3,20-dione (I), m.p. 215°–222° (dec.); $[\alpha]_D -62°$ (c, 1.0 in dioxane); $\lambda_{\text{max.}}^{\text{MeOH}}$ 243 mμ ($\varepsilon=16{,}000$): $\lambda_{\text{max.}}^{\text{KBr}}$ 3.00 μ (OH): 5.80 μ (20-keto); 5.94 μ and 6.16 μ (Δ^4-3-keto). *Anal.* Found: C, 69.48; H, 8.30. Calcd. for $C_{21}H_{30}O_5$: C, 69.58; H, 8.34%.

We also obtained a polymorphic modification1) of (I), infrared spectra of which, $\lambda_{\text{max.}}^{\text{KBr}}$ were 2.84 μ, 5.82 μ, 5.89 μ, 6.01 μ and 6.19 μ.

The 2,21-diacetate of (I) was obtained with acetic anhydride and pyridine, m.p. 215°–218°; $[\alpha]_D +7°$ (c, 1.0 in dioxane); $\lambda_{\text{max.}}^{\text{MeOH}}$ 244 mμ ($\varepsilon=15{,}000$); $\lambda_{\text{max.}}^{\text{KBr}}$ 2.84, 2.96 μ (OH), 5.72 μ (acetate carbonyl), 5.80 μ (20-keto), 5.98 μ, 6.18 μ (Δ^4-3-keto), 8.08 μ and 8.25 μ (c-o-c). *Anal.* Found: C, 67.43; H, 7.59. Calcd. for $C_{25}H_{34}O_7$: C, 67.24; H, 7.68%.

The properties of the isolated compound and its acetyl derivative were in agreement with those reportes for 2β, 17α, 21-Trihydroxy-4-pregnene-3,20-dione1).

Crystallization of the middle fractions afforded 11β, 17α, 21-Trihydroxy-4-pregnene-3, 20-dione (II) (Hydrocortisone), m.p. 201°–206°; $[\alpha]_D +160°$ (c, 0.5 in alc.); $\lambda_{\text{max.}}^{\text{KBr}}$ 2.92 μ (OH), 5.80 μ (20-keto), 6.09 μ and 6.20 μ (Δ^4-3-keto). *Anal.* Found: C, 69.52; H, 8.42. Calcd. for $C_{21}H_{30}O_5$: C, 69.58; H, 8.34%. The 21-monoacetate of (II), m.p. 212°–220°; $[\alpha]_D +163°$ (c, 0.5 in alc.).

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1) H. L. Herzog, *J. Am. Chem. Soc.*, **79**, 3921 (1957).
2) G. Greenspan et al., *ibid.*, **79**, 3922 (1957)

In addition to these constants, their infrared spectra and paper chromatographic behaviors agreed with those of the authentic samples of hydrocortisone and its monoacetate.

The crystallization of the last fractions afforded a small amount of 11α, 17α, 21-Trihydroxy-4-pregnene-3, 20-dione (III), m.p. 205°–209°; $[\alpha]_D+115°$ (c, 1.0 in alc.); $\lambda_{\text{max.}}^{\text{KBr}}$ 2.95 μ (OH), 5.84 μ (20-keto), 6.08 μ and 6.22 μ (Δ^4-3-keto). *Anal.* Found: C, 69.40; H, 8.50. Calcd. for $C_{21}H_{30}O_5$: C, 69.58; H, 8.34%.

The infrared spectra and paper chromatographic behaviors agreed with those of authentic samples.

In the case of *Sclerotinia sclerotiorum*, identical results were obtained.

Hydroxylation of progesterone and 17α-Hydroxyprogesterone produced by these microorganisms are now under investigation.

We are greatly indebted to Dr. K. Tsuda, Dr. T. Asai, Professors of the University of Tokyo and Mr. M. Matsui, Director of this laboratory for their helpful advice and constant guidance in this work.

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(in Japanese)
Vol. 32, No. 5 (1958)

On the Inhibitor for Starch Accumulation in the Root of *Beta vulgaris*. Part VI. Isolation and Identification of Oleanolic Acid from Lime Cake in Carbonation Process. (p. *331～333*)

By Yataro OBATA and Yoshinori ISHIKAWA
(*Department of Agricultural Chemistry, Faculty of Agriculture, Hokkaido University*)

The presence of phosphorylase inhibitor in the root of sugar beet and mangold was previously reported by the author. The inhibitor, which was isolated and purified from the sugar beet juice, was shown previously to be a triterpenoid saponin by its positive Liebermann-Burchard reaction.

In a sugar beet factory, a remarkable phosphorylase inhibiting action is observed in diffusion juice but not in thin juice. Therefore, it is clear that the inhibitor is eliminated by the liming and carbonating process. The lime cake shows reddish violet coloration of Liebermann-Burchard reaction indicating the presence of triterpenoid saponin.

The lime cake was extracted by refluxing with 50% ethyl alcohol containing 2 N sulfuric acid and for 1 hour. The alcohol extract was concentrated in vacuo and the precipitate formed was hydrolysed with 2 N sulfuric acid for 40 hours. The sapogenin was purified through ammonium salt, potassium salt, and identified to be oleanolic acid, $C_{30}H_{48}O_3$, m. p. 304～305°. The infrared spectrum of the purified sapogenin was coincided with oleanolic acid separated from mistletoe, sugar beet and mangold. From the sapogenin, methyl ester m. p. 197°, acetate m. p. 259～260°, and acetate of the methyl ester m. p. 215～216° were prepared which showed no depression of melting points when mixed with authentic specimens of those oleanolic acid derivatives.

Studies on the Form of Iron and Iron-containing Material in the Living Tissue. Part XVI. Studies on Conalbumin. (2) (p. *337～340*)

By Masao KANAMORI and Ichiro MAEDA
(*College of Agriculture, Saikyo Uuiversity*)

We have studied the electrophoretic analysis of the whole egg white proteins, by the ammonium sulfate fractionation procedure, and recognized that the conalbumin prepared by the Alderton's method, gave electrophoretic pattern, and after dialysis for 3 days at acid pH values it was converted to two components. The isolated conalbumin was colorless and did not combine with the yellow pigment (riboflavin and it's derivatives) of egg white, and it was modified to two components electrophoretically by the acid modification.

We have recognized that yellow proteins of egg white concentrated into the portion which was precipitated by 2.6 mol saturation of ammonium sulfate, and yellow pigment was released easily from the yellow protein at acid pH values (below pH 4.0) and combined tightly with its protein portion at alkaline pH values. Moreover the yellow pigment of yellow protein fraction in egg white was mainly riboflavin, and its protein portion was ovomucoid which can be combined with riboflavin, flavinmononucleotide, flavindinucleotide, by means of spectrophotometric, electrophoretic and paper-chromatographic analysis.

Studies on the Carbohydrates of Rape-seed. (p. *340～343*)

By Takashi MIZUNO
(*Department of Agricultural Chemistry, Faculty of Agriculture, Shizuoka University*)

The qualitative and quantitative analysis of free sugars, the constructive sugars and their molecular ratios of polysaccharides isolated fractionality from rape-seed were investigated by paper chromatography.

The obtained results were summerized as follows:

Fructose 0.51%, glucose 0.21%, saccharose 1.11%, raffinose 0.15% and stachyose 0.19% for the defatted water-free rape-seed.

Hot water soluble-polysaccharide; arabinose: galactose: ribose=7:7:1.

Hot 0.5% ammonium oxalate soluble-polysaccharide; arabinose: ribose: galacturonic acid: galactose=4:2:2:1.

Cold 5% NaOH soluble-polysaccharide; arabinose: glucose: xylose: ribose: rhamnose=12:5:5:4:1.

Hot 5% NaOH soluble-polysaccharide; glucose: xylose: arabinose: ribose=1.7:1.4:1.4:1.0.

Fundamental Studies on the Aerobic Fermentation. Part VI. On the power Requirements of Aeration and Agitation in Fermentors. (p. *344～348*)

By Koichi YAMADA and Jyoji TAKAHASHI

(*Department of Agricultural Chemistry, Faculty of Agriculture, Tokyo University*)

In the aerobic culture, oxygen absorption coefficient K_d has a great influence on the growth and an amount of fermentation products of microorganisms.

The consumptions of the power in aerations and agitations which are required to provide aerobic cultures with some values of K_d are determined in three types of laboratory fermentors.

The requirements of the power for agitations are determined by means of a torque table, and those of aerations are determined theoretically.

Based on the relations between K_d and total power consumption, (aeration power+agitation power), the most suitable aeration rates and agitator speeds for minimizing the total power in consumptions which are required to supply an aerobic culture with optimum K_d are discussed.

Studies on the Ammonia Assimilation by Yeast. Part I. Fundamental Conditions in Primary Ammonia Assimilation by Nitrogen Starved Yeast. (p. *348～352*)

By Kiyoshi SUGAWARA, Kazuo KUMAGAI and Kensuke SHIMURA

(*Laboratory of Biochemistry, Faculty of Agriculture, Tohoku University, Sendai, Japan*)

Ammonia assimilation by *T. utilis*, which had been starved from nitrogen source for 5 hrs., was studied. A linear relationship was observed between ammonia levels of the medium and its assimilation during 20 min. shaking in the presence of glucose. However in a short time (5 min.) experiment in the absence of glucose, their relation was given by Freundlich's adsorption formula, $X=kc^{\frac{1}{n}}$. The uptake of nitrogen was found in the optimum at 25° to 30° and was not significantly affected by pH of the medium in the range from 3.4 to 7.2. The ammonia nitrogen taken into the cell was shown to be mainly assimilated to glutamine, glutamic acid, alanine and furthermore to acid soluble nucleotides, and later to protein. Comparing the nitrogen assimilations of 4 hrs. nitrogen-starved yeast with those of 12 hrs. starved cells, it was concluded that some level of soluble nitrogen was necessary for protein formation. In the anaerobic conditions, the formation of soluble nitrogen was inhibited more remarkably than that of the insoluble fraction, probably protein nitrogen.

Studies on the Ammonia Assimilation by Yeast. Part II. Effects of Organic Acids. (p. *352～355*)

By Kiyoshi SUGAWARA, Kazuo KUMAGAI and Kensuke SHIMURA

(*Laboratory of Biochemistry, Faculty of Agriculture, Tohoku University, Sendai, Japan*)

The effects of several organic acids and other carbon sources on the ammonia assimilation by nitrogen-starved *T. utilis* were studied. A slightly stimulating effect was observed with α-ketoglutarate and pyruvate. On the contrary, short chain fatty acids such as formate, acetate, butyrate and valerate inhibited markedly. Other organic acids participating in TCA cycle did not reveal significant effects in the presence of sufficient glucose, but under the low glucose level, stimulating effects were observed. The addition of organic acids increased the intracellular soluble nitrogen as well as the ammonia uptake. Inhibitory action of short chain fatty acids was demonstrated to occur at the stage of transformation of the intracellular soluble nitrogen to protein nitrogen.

The Formation of Amino Acids in Ki-moto. Part I. On the Extraction of Nitrogenous Compounds in Moto Mash. (p. *355～359*)

By Hiroichi AKIYAMA

(*The Brewing Experiment Station, Tax Administration Agency*)

The author has been studied the changes of nitrogen-compounds in Ki-moto, the classical method of yeast inocculum[1], and in this paper reports the extraction method of N-compounds to discuss the protein digestion during the first stage of Moto-making. The following results were obtained:

1) A comparative extraction of Moto mash was made with various acids, buffer, alkali, inorganic salts and alcohol. In the first stage of Moto-making, a N-compound was produced which was water-insoluble, acid-soluble and precipitated by trichloroacetic acid. Moreover this fraction was readily decomposed by protease in acid side about pH 3.

2) For extraction of N-compounds in Moto mash water, 0.1 and 0.5% lactic acid solution should be used.

3) Folin's colorimetric method gave good results in place of Kjeldahl method in N-determination in Moto mash.

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[1] K. Arima et al., Yeasts, Dr. W. Junk. Publishers, The Hague, 1957.

On the Assimilation of Nitrites by Fungi. Part IV. (p. *366~371*)

By Yoshishige OKAZAWA and Akira KUNINAKA

(*Department of Agricultural Chemistry, Faculty of Agriculture, of University Tokyo*)

Aspergillus molds exhibit a wide variation in their abilities of nitrite assimilation(1~4). The authors studied the causes of this variation.

Aspergillus molds are classified to six types by the difference in growth on the acidified Sakaguchi-Wang's medium, which contains sodium nitrite as its sole nitrogen source. The variation in ability of nitrite assimilation depends partly on sensitivity of mold to undissociated nitrous acid molecule.

Aspergillus molds are also classified to seven types by the difference in abilities of assimilation of various nitrogen sources. There are no special relations between the abilities to assimilate nitrite and that to resist to hydroxylamine. The strains unable to grow on both Czapek's and Sakaguchi-Wang's media, require ammonium salts or amino acids as the indispensable nitrogen sources.

After all the strains unable to grow on Sakaguchi-Wang's medium are impossible to produce nitrite-reductase in the absence of any other nitrogen sources than nitrites. On the other hand, the strains that are able to grow on Sakaguchi-Wang's medium are able to produce nitrite-reductase under such conditions.

(1) K. Sakaguchi and Y. Wang, *J. Agr. Chem. Soc. Japan*, **10**, 459 (1934).
(2) K. Sakaguchi and Y. Wang, *J. Agr. Chem. Soc. Japan*, **12**, 59 (1936).
(3) K. Sakaguchi and Y. Wang, *J. Agr. Chem. Soc. Japan*, **12**, 63 (1936).
(4) Y. Wang, *J. Agr. Chem. Soc. Japan*, **12**, 877 1936).

Studies on Bacterial Proteinase. Part X. Proteolytic Action of a Neutral Proteinase of *Bacillus subtilis*. (p. *375~379*)

By Juichiro FUKUMOTO, Takehiko YAMAMOTO and Kazuhiro ICHIKAWA

(*Institute of Polytechnics, Osaka City University*)

The neutral proteinase of *Bacillus subtilis*, which had been obtained as an electrophoretically pure preparation, hydrolyzed gelatin more actively than trypsin did up to the reaction stage of 50% reduction in viscosity of gelatin solution, but thereafter the former became fairly less active than the latter in the gelatin hydrolysis. The bacterial proteinase could easily attack milk casein, hemoglobin and soybean protein, making them trichloroacetic acid-soluble (by a modification of Folin's method), although the degree to hydrolyze the peptide bonds of the substrates was only 35% or so in the maximum. The activity on egg albumin as the substrate, however, was very small in all the activity-assay methods applied in this experiment. The paper chromatograms of the proteinase-digests of casein and gelatin prepared by use of a large amount of enzyme, revealed the enzyme to release leucine, glycine and glutamic acid from the substrates. Muscle treated with the enzyme gave a more or less friable mass, suggesting that the proteinase attacks the supporting tissue more easily than the fibrils.

Studies on the Effects of Dextran manufactured in Japan on the Physiological Functions. Part I. The Effects of Dextran on the Physiological Function of Erythrocytes. (p. *379~383*)

By Takehiko KAWANO

(*Research Laboratory, The Blood Plasma Corporation of Japan*)

In Japan it was begun in 1954 to manufacture dextran as a plasma volume expander, and the study on the effects of the Japanese dextran on the physiological function was considered an important problem because the differences of the properties among American, English and Swedish dextran had been already pointed out.

The author reports in this paper the effects of Japanese dextran on the physiological function of erythrocytes in the first part of the series, and next the data obtained about erythrocyte sedimentation rate, false agglutination, the preservation of erythrocytes and the oxygen carrying capacity of blood are shown.

The author describes the conclusion as follows;

(1) The Japanese dextran does not increase erythrocyte sedimentation rate and bring about false agglutination: therefore, it is not conceivable that the dextran can inhibits the function of erythrocyte directly.

(2) The bad effect of the dextran on the preservation of erythrocytes is not observed, and this fact seems to show that the dextran is effectless on the metabolism of erythrocyte in vitro.

(3) In vitro the efficiency of oxygen saturation of blood decreases slightly when mixed with the dextran, but it is not clear whether the dextran inhibits the oxygen carrying capacity of blood substantially or not. In vivo it seems to be difficult to expect the same results, and about this point discussion will appear in the later paper of this series (Part III).

Studies on Chitin from the Standpoints of Polymer Chemistry. Part VI. Physicochemical Behavior of Carboxymethyl Chitosan as Polyampholytes (1) Molecular Weight (Light Scattering). (p. *383~386*)

By Satoshi OKIMASU

(*Hiroshima Women's College*)

The molecular weights of two sorts of carboxymethyl chitosan salts, which were prepared through carboxymethylation and deacetylation of crab chitin following the method reported previously by the author, were measured using a Brice-Phoenix Light Scattering Photometer and Differential Refractometer, and they proved to be 2.80×10^5 on an average. The reduced turbidity versus concentration curves show the same upward tendency with dilution of the solution as that of ordinary polyelectrolytes in water, but some aggregation of polymer ions is observed in the presence of 0.1 N NaCl. Thus, it is clear that carboxymethyl chitosan is one of the typical polyelectrolytes, and the molecular weight of natural chitin is large in almost same order as that of natural cellulose.

Studies on Chitin from the Standpoints of Polymer Chemistry. Part VII. Physicochemical Behavior of Carboxymethyl Chitosan as Polyampholytes (2) Stability of the Solution. (p. *387~389*)

By Satoshi OKIMASU

(*Hiroshima Women's College*)

As some of the preliminary experiments to investigate the physicochemical behavior of carboxymethyl chitosan salts, several properties related to stability of the aqueous solution were examined: (1) When the solution, especially of sodium salts, is offered to dialysis, a remarkable membrane hydrolysis is observed. (2) Viscosity of the solution falls gradually with time at the room temperature, but after about thirty hours it becomes equilibrium. (3) According to the results obtained through the coagulation experiments of the solution with 99% ethanol at various conditions, it is clear that stability of the solution is supported mainly with effective charges regardless of non-electric solvation of polymer ions.

On the Migration Distance in Paper Electrophoresis. Part VI. Studies on the Migration Distance of Dyes. (p. *390~399*)

By Kiyoshi SAKAMOTO and Kinuko SAITO

(*Laboratory of Nutrition, Faculty of Junior Course, Kagoshima Prefectural University*)

The authors studied the migration distance of dyes in the apparatus of paper-electrophoresis fixed with the filterpaper held horizontal and with the evaporation from the paper strips being admitted.

They have found that dyes were separated into several groups by the capillary analysis and that the displacement ratio R-values in the occasion had close relationship with the migration distance in the apparatus of paper-electrophoresis, in other words, the dyes with similar R-values in capillary analysis had almost parallel graphic lines between the axes of dropped points from the center of filterpaper and migration distance. In assorting dyes with similar R-values into several groups, each of then obtained MG values. And they affirmed the fact by experimental measurement and mathematical analysis that these values, as in the case of amino acids and serum proteins above, were less influenced by the migrating circumstances.

On the other hand, they have also investigated the relation of groups of dyes and their functional radicals with the migrating direction, and found the fact that the decrement of the migration velosity of these dyes was approximately similar to the logarithmic decrement at each definite interval.

Studies on Streptomycetes. Assay of Vitamin B_{12} in the Presence of Dihydrostreptomycin. (p. *399~405*)

By Kôichi NAKAZAWA, Motoo SHIBATA, Hiroichi YAMAMOTO, Eiji HIGASHIDE, and Toshihiko KANZAKI

(*Institute for Fermentation, Osaka*)

The authors found that *Streptomyces humidus* nov. sp. produces vitamin B_{12} besides dihydrostreptomycin. They investigated the assay method of vitamin B_{12} existing together with dihydrostreptomycin in the fermented broth and found that microbioassay was the best method for assaying small quantities of vitamin B_{12}.

As to the test microorganism *Escherichia coli* mutant was suitable for speedy assay. Vitamin B_{12} could be assayed by this method in the presence of one γ per ml. of dihydrostreptomycin. However, when the microbioassay is conducted in the presence of more concentrated dihydrostreptomycin, a *Escherichia coli* mutant resistant to one thousand γ per ml. of dihydrostreptomycin was produced, but as the mutant was less growing in the normal assay medium, the medium was modified as follows: K_2HPO_4, 14 g; KH_2PO_4, 6 g; $(NH_4)_2SO_4$, 2 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; citric acid, 0.8 g; NaCl, 5 g; glucose, 4 g; *l*-asparagine, 4 g; *dl*-tryptophane, 400 mg; *l*-glutamic acid, 400 mg;

l-arginine hydrochloride, 400 mg; *l*-histidine hydrochloride, 400 mg; glycine, 400 mg; *l*-proline 400 mg; distilled water, 1000 ml.

Using this medium vitamin B_{12} could be assayed in the presence of one thousand γ per ml. of dihydrostreptomycin. Incidentally, *Escherichia coli* mutant requires vitamin B_{12} or *dl*-methionine for its growth.

Bacteriophage Phenomena in Fermentative Microorganisms. Part IV. Isolation of Bacteriophages Causing the Troubles in the Production of Bacterial Amylase. (p. *405～409*)

By Kakuo KITAHARA and Takichi KANEKO

(*Institute of Applied Microbiology, University of Tokyo*)

Plants in Japan which produce amylase with the use of bacteria of *Bacillus subtilis* group had been sometimes suffered from occasional bacterial lysis. To clarify the nature of the lysis, the lysates sent from the plants were examined for the bacteriolytic activity. It was demonstrated that the lysates from three different plants contained specific bacteriophages P 1, P 2, and P 3, and one of these bacterial strains having been industrially used was lysogenic (*B. subtilis* K for phage PK). Some probable mutants of these phages were also isolated. The bacteriophages of the type were able to lyse the cells even in their stationary growth phase. From these findings it was concluded that the bacterial lysis in amylase plants was mainly caused, by bacteriophage.

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Bacteriophage Phenomena in Fermentative Microorganisms. Part V. Characterization of Phages Active against Amylase-Producing Bacteria. (p. *411～414*)

By Kakuo KITAHARA and Takichi KANEKO

(*Institute of Applied Microbiology, University of Tokyo*)

General characters of the bacteriophages, the isolation of which was reported in the preceding paper, were described. Strains P 1, P 2, P 3, and PK were serologically distinct and had different host ranges. P 1 T, a mutant of P 1, had a host range much broader than the wild type. PK 10 and PK 7 were concluded to be clear plaque mutants of temperate PK. All these strains had a tendency of preferably attacking amylase producers, but some other strains of *Bacillus subtilis* were also sensitive to some of them. Mutants of *B. subtilis* H were obtained which were resistant to one of P 1, P 1 T, and P 3. PK 10 and PK 7 when they act upon *B. subtilis* H gave the rise of lysogenic strains, but not typical resistant ones. All these phage-resistant and lysogenic strains were not inferior to the wild in amylase productivity.

Steroids and Microbes. Part IV. Studies on the Antibiotic Action of Steroids. (p. *415～419*)

By Yuichiro KUROSAWA

(*Tsurumi Chemical Research Institute, Tsurumi, Yokohama*)

Steroid compounds (cholesterol, cholesteryl acetate, cholesteryl chloride, cholesteryl hemisuccinate, cholesteryl 5, 6α-oxide, cholesteryl 5, 6β-oxide, Δ^4-cholestenone, 6β-Cl-cholestane-3β, 5α-diol, 5-Cl-cholestane-3β, 6β-diol-3-monoacetate, cholestane-3β, 5α, 6β-triol, *i*-cholesterol, *i*-cholesteryl methylether, testosterone, methyltestosterone, estrone, estradiol, estradiol-17-thioglycolic acid ester, ethinylestradiol, pregnenolone, progesterone, ethinyltestosterone and desoxycorticosterone) were tested for antibiotic activity against *Aspergillus niger*, *Penicillium* sp., *Saccharomyces chevalieri*, *Mycoderma chevalieri*, *Torulopsis candida*, *Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis*, *Pseudomonas fluorescens*, *Bacillus thiaminolyticus* Matsukawa, *Sarcina* sp., and *Mycobacterium tuberclosis* 607 by the serial dilution method in a nutrient broth of the following composition: Czapek-Dox containing 0.2% of Difco yeast extract (glucose 40 g, $NaNO_3$ 3.0 g, K_2HPO_4 1.0 g, KCl 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.05 g, $FeSO_4 \cdot 7H_2O$ 0.01 g, distilled water 1 l), Bouillon (meat extract 10 g, peptone 10 g, NaCl 1.5 g, dist. water 1 l), and Bouillon containing 3% of glycerol. From the result, it was found that cholestane-3β, 5α, 6β-triol, ethinylestradiol and desoxycorticosterone were effective in inhibiting the growth of *Staph. aureus*, *Baci. subtilis*, *Pseudomonas fluorescens*, *Baci. thiaminolyticus* Matsukawa, *Sarcina* sp., and *Mycobact. tuberclosis* 607 at concentrations of 0.01-1 mg/ml.

On the Discoloration Phenomena of the Conidia of *Aspergilli*.

Metabolic Product of *o*-, *m*-, methoxy-benzoic acid, and *p*-ethoxy-benzoic acid by *Aspergillus oryzae* and *Aspergillus flavus*. (p. *419~422*)

By Yuichiro KUROSAWA

(*Tsurumi Chemical Research Institute, Tsurumi, Yokohama*)

Up to the present day the author has been studying the antifungal activity of many sorts of spices against *Aspergillus oryzae*, *Penicillium* sp., and *Trichophyton mentagrophytes*, and noticed that Aubepine-crystal (brought from Toyodama Spice Co., Ltd., whose m. p. was 38–39° and the mixed m. p. with synthetic *p*-methoxy-acetophenone was not depressed) could be changed the color of conidia of *Asp. oryzae* from green to pink. Similarly, the color of conidia of *Asp. flavus* was changed into pink.

Anisic acid (*p*-methoxy-benzoic acid m. p. 184°) changed the color of conidia of *Asp. oryzae* into pink, but did not change the color of conidia of *Asp. flavus*. In this case, anisic acid was metabolized to hydroquinone-monomethylether by *Asp. oryzae* and to *p*-hydroxy-benzoic acid by *Asp. flavus*. However, *o*-, *m*-, methoxy-benzoic acid was not so easily changed as it is compared with anisic acid.

And then, *p*-ethoxy-benzoic acid (m. p. 196–7°) changed the color of conidia of *Asp. oryzae* into pink faintly, but did not change the color of conidia of *Asp. flavus*. In this case, *p*-ethoxy-benzoic acid was metabolized to hydroquinone-monoethylether by *Asp. oryzae* and *p*-hydroxy-benzoic acid by *Asp. flavus*.

Protease Production by the Submerged Culture of *Aspergillus oryzae*. Part I.

Selection of Strain and Medium Suitable for Producing Protease Active in Neutral and Alkaline Reaction (1). (p. *422~426*)

By Kazuo MOTONAGA and Yukichi MIURA

(*Takamine Laboratory, Sankyo Co., Ltd.*)

This study was carried out to obtain broth containing high yields of protease, active in neutral and alkaline reaction, at the smallest content of impurities by the submerged culture of *Asp. oryzae*.

Among tested 150 strains of *Asp. oryzae* in our collection, the strain No. 433 was found to produce the highest yield of protease.

Media was investigated on protease production by the strain No. 433 and the following was found to be most suitable:

glucose	1.0 %
peptone	0.42%
KH_2PO_4	3.0 %
$MgSO_4 \cdot 7H_2O$	0.01%
initial pH	7.5 (by NaOH)

This medium was compared with several media, containing protein or not, for protease production using the strain No. 433 and another, and it seemed to be the best medium.

Studies on Denatured Egg Albumin. Part II.

Viscosimetric Studies on the Aggregation of Denatured Egg Albumin. (p. *426~430*)

By Hiromu SHIMOMURA and Daizo YONEZAWA

(*Faculty of Agriculture, University of Osaka Prefecture*)

By observing the change of the viscosity of the solution under various conditions, the authors investigated the factors which control the aggregation of denatured egg albumin molecules in solution. The denatured egg albumin was prepared by warming the acidified egg albumin solution at 44.4°C for 5 hrs. and purified by repeating the isoelectric precipitation after the method of Mac Pherson et. al.[1]. The viscosity measurement was made at 5°C with Ostwald's viscosimeter. It was observed that the change of the viscosity of the denatured egg albumin solution during storage was highly sensitive to the pH and ionic strength of the solution. The high ionic strength accelerated the increase of the viscosity. The viscosimetrically stable solutions were obtained at ionic strength 0.05 in the case of pH 3.3 and at about 0.01 in the case of pH 4.0, the relative viscosities of which were of same order in both. (about 1.4 in 1% solution). The concentration of denatured albumin affected the change of the viscosity somewhat. The effects of warming and irradiation of ultra-sonic waves were also investigated.

[1] C. F. C. Mac Pherson & M. Heidelberger, *J. Amer. Chem. Soc.*, **67**, 574 (1945)

Studies on the Bamboo Pulp. Part II.

The pulping of Bamboo by the Kraft Process. (1) On the Sulfidity. (p. *430~432*)

By Kazuki ÔNO and Tetsuzo TÔNO

(*Faculty of Agriculture, University of Osaka Prefecture*)

The bamboo chip is not suited to acidic cooking but to alkaline cooking for its high content of water soluble matter and especially of pentosan in the stem. Therefore, the sulphate cooking has been studied in this work. First, the effect of sulfidity was investigated.

The Japanese bamboo, Kutiku (*Phyllostachys reticulata*) was cooked for two hours at 175°C., the sulfidity being varied within the range from 0 to 30% and the dosage of cooking chemicals being 18, 19 and 20% (effective alkali expressed as NaOH) per chip respectively.

Considering the yield and the delignification quality, the result of the investigation was examined. The effect appeared already at the 15% level of sulfidity, and at the 20% level the optimum result was obtained within the above mentioned range of cooking chemicals. The yield, residual lignin content and permanganate number of unbleached bamboo pulp were 38–40%, 3–5% and 16–20 respectively at this optimal sulfidity.

Studies on the Pigments of Horsetail, Spore Stalk of *Equisetum arvense* L. Part I. Isolation of Two Yellow Glycoside Pigments. (p. *436～439*)

By Toshio NAKABAYASHI

(*Department of Agricultural Chemistry, Faculty of Agriculture, Shizuoka University*)

In the course of the studies on the thermostable antithiamine factor of vegetables, two glycoside pigments, found only in the spore, were isolated in the yellow crystalline state from the horsetail as the antithiamine factors. These two pigments named as articulation (A) and isoarticulation (I) were hydrolyzed to glucose and aglycones articulation (a) and isoarticulation (i) when they are boiled with 20% sulfuric acid.

The properties of pigments were as follows:

	A ($C_{21}H_{22}O_{13} \cdot 2H_2O$)	a ($C_{15}H_{12}O_8 \cdot 2\frac{1}{2}H_2O$)	I	i
		Physical Properties		
crystalline (m.p.)	yellow needle (227°)	yellow needle (293°)	yellow needle (226°)	greenish brown needle (266°)
acetate (m.p.)	colorless needle (218～220°)	colorless needle (213～217°)	colorless needle (206～208°)	colorless needle (189～191°)
absorption maximum (mμ)	262,346,389.	262,277,339,386.	225,249,275,312,335,385.	227,275,333,383.
		Color Change in Alcoholic Solution		
by NaOH	orange yellow	blue	orange yellow	blue
by $FeCl_3$	greenish brown	dark blue	dark blue	dark blue
by $MgAc_2$	orange yellow	blue	orange yellow	blue

On these pigments, HCl-MG reaction was negative but strong yellow color was developed by $AlCl_3$, and especially aglycones had strong reducing activity. From these analytical results, it was supposed that these pigments were not ordinary flavonoid but some new pigments having the peculiar structure.

Studies on the Higher Fatty Alcohols. Part XI. Preparation and Properties of Some Ethenoxylate Unsaturated Higher Fatty Alcohols. (p. *440～443*)

By Shinroku MASUYAMA

(*The Osaka Municipal Technical Research Institute*)

Polyethylene glycol ethers of soybean, corn, sardine, and walnut fatty alcohols have been prepared by reaction of the alcohol with ethylene oxide at atmospheric pressure in the presence of a basic catalyst.

The surface-active and related properties of the ethenoxylated unsaturated fatty alcohols, including surface tension, acid stability, wetting and emulsification were measured.

Ethenoxylated products appeared to be the excellent emulsifying agents of the vegetable oils.

Studies on the Higher Fatty Alcohols. Part XII. Some Citric Acid Monoesters of Higher Fatty Alcohol as Antioxidants. (p. *443～445*)

By Shinroku MASUYAMA and Satoshi YASUHARA

(*The Osaka Municipal Technical Research Institute*)

Citric acid monoesters of dodecyl, octadecyl, linol, cotton, and corn fatty alcohols have been prepared and some of their physical constant determined.

These compounds have the essential structure for effective chelation of metals.

Cottonseed oil was stabilized with mixtures of BHA, PG, and unsaturated fatty alcohol monoesters; and BHA, PG, and citric acid under laboratory conditions.

No particular significance was attached to the difference between synergistic effects of unsaturated fatty alcohol monoesters and citric acid.

Studies on Growth Inhibition on Hiochi-bacteria, Specific Saprophytes of Sake. Part I.

Estimation Method of Antibiotic Activity against Hiochi-bacteria. (p. *445~450*)

By Seiji NAKAMURA and Teruo SHIRO

(*Yamamura Sake-brewing Laboratory*)

Twenty antibiotic substances were estimated their activities against hiochi-bacteria by the under described methods. Homomycin, mikamycin, chloramphenicol and antinomycin inhibited the growth of hiochi-bacteria. Penicilline-V was more inhibitory than penicilline-G to the growth of hiochi-bacteria.

In order to estimate the activity of antibiotics against hiochi-bacteria, the following three methods were applied.

1) Dilution method. Matsuyama's sake medium(1) containing 1% polypeptone is used as preliminary culture and assay media. Preliminary culture carried out incubating for 7 days at 30°C, and then, after further 7 days the degree of growth of bacteria is observed in assay medium.

2) Vertical diffusion method. Pour 5 ml of Matsuyama's ager medium(1), which contained methylene blue and was suspended with hiochi-bacteria, into test tube and cool. Then 1 ml of the solution of active substance is placed upon the agar, the surface of its layer is covered with 1 ml of liquid paraffin. After 3 days at 30°C, the depth of colour faded zone is measured for estimating the antibiotic activity.

3) Agar cup method. 8 ml of the same agar medium suspended with hiochi-bacteria as vertical method is poured plate, and covered by 20 ml of 1.5% agar solution. The etsimation of potency is determined in the same way as the ordinary cup method after incubation for 3 days at 30°C.

1) A. Matsuyama, *J. Agr. Chem. Soc. Japan*, **28**, 418, 422 (1954).

Studies on Growth Inhibition of Hiochi-bacteria, Specific Saprophytes of Sake. Part II.

Screening of Aspergilli and Streptomyces. (p. *450~454*)

By Seiji NAKAMURA, Teruo SHIRO and Noriyuki KUNISADA

(*Yamamura Sake-brewing Laboratory*)

The authors searched for antibiotics active against hiochi-bacteria by shaking culture and by Koji-making among 314 strains of Aspergilli. The culture filterates of 42 strains showed inhibition against the growth of hiochi-lactbacilli H-34(1), but 130 strains showed no inhibition, and remaining 147 strains, on the contrary, stimulated the growth of H-34. Almost all of strains those showed activity against H-34 also inhibited the growth of true hiochi-bacteria H-1(1), whereas very few Aspergilli inhibited the growth of Teramoto's hiochi-bacteria T-101(2). As the result, only 4 strains showed antibiotic activity against the 3 strains of hiochi-bacteria.

On Koji-making (culture of Aspergilli on steamed rice), almost all their extract by N/10-lactate buffer (pH 4.5) stimulated the growth of 3 strains of hiochi-bacteria, but only 6 strains of Aspergilli completely inhibited the growth of H-1 and H-34, although they had a few antibiotic activity against T-101. Nevertheless, in the case of shaking culture, filtrates of these six strains of Aspergilli did not inhibit the growth of hiochi-bacteria.

On the other hand, the authors obtained 5 strains of Streptomyces which showed activity against hiochi-bacteria among 99 strains. They had always high activity against the 3 strains of hiochi-bacteria under any cultural conditions. From these results, it seems to be the antibiotics produced by Aspergilli might be differ from those of Streptomyces.

1) K. Kitahara et al., *J. Gen. Appl. Microbiol.*, **3**, 102, 111 (1957).

2) S. Teramoto et al. *J. Ferm. Tech.*, **32**, 180 (1954).

Studies on the Carbohydrates of Tobacco. Part III.

Carbohydrates in the Stem and Root of Tobacco. (p. *467~470*)

By Takashi MIZUNO, Senzi KAINUMA, Akira NAKAGAWA, and Tadao KINPYÔ

(*Biochemical Laboratory, Department of Agricultural Chemistry, Faculty of Agriculture, Shizuoka University*)

Free sugar and various polysaccharides which were contained in the stem and root of Japanese bright yellow tobacco were investigated, and the following results were obtained:

The stem contained reducing sugar (composed of glucose and fructose) 2.41%, and non-reducing sugar (mainly saccharose) 3.79% on the dry basis; the root contained fructose plus glucose 0.18% and saccharose 3.10%.

Polysaccharides were isolated from the stem and root portions of tobacco plant and fractionated into hot 50% ethanol soluble, cold and hot water soluble, hot 0.5% ammonium oxalate soluble, cold 5% sodium hydroxide soluble (separated into two fractions: precipitating with acetic acid and non-precipitating with acetic acid but precipitating with ethanol), hot 5% sodium hydroxide soluble (separated into two fractions as in the above procedure), cold 20% sodium hydroxide soluble (β- and γ-cellulose fraction) and cold 20% sodium hydroxide insoluble polysaccharide (α-cellulose fraction).

Chemical and physical properties of each of the above isolated polysaccharides were made clear, both with the constructive sugar that were investigated.

Studies on Chitin from the Standpoints of Polymer Chemistry. Part VIII. Physicochemical Behavior of Carboxymethyl Chitosan as Polyampholytes (3). Viscosity of the Solution. (p. *471~475*)

By Satoshi OKIMASU

(*Hiroshima Women's College*)

The reduced viscosities of aqueous solution of carboxymethyl chitosan salts, that were measured at various velocity gradients in the capillary viscometers, change delicately in almost same manner as that of ordinary polyelectrolytes at various conditions of the solution, except that an isoelectric point is observed clearly at the changes with pH. That is, the reduced viscosity versus concentration relationships conform well to Fuoss' equation. One of the remarkable characteristics of the solution is a very strong structural viscosity, which is considered to be due to a net work structure among polymer ions as well as a resistance for the deformation. It seems that the net work structure is unstable and changes gradually to the ion distribution of ordinary polyelectrolytes in a dilute solution. In a relatively concentrated solution, however, the structure remains as it is, and has a strong effect upon the structural viscosity.

Studies on Chitin from the Stundpoints of Polymer Chemistry. Part IX. Physicochemical Behavior of Carboxymethyl Chitosan as Polyampholytes (4). Conductance of the Solution. (p. *475~478*)

By Satoshi OKIMASU

(*Hiroshima Women's College*)

The effects of the concentration of colloid, added neutral salts and pH on the equivalent conductivity of aqueous solution of carboxymethyl chitosan salts are similar well to the effects on that of ordinary polyelectrolytes, and also coincide well with the results of both coagulation experiments and viscosity measurements of carboxymethyl chitosan reported previously. In a relatively concentrated solution, however, the interference of conductance with a special net work structure is observed. Some clear dissimilarity is also observed among both hydrochloric acid and sodium salts of carboxymethyl chitosan on the effects of added sodium chloride upon the equivalent conductivity. Moreover, the fact that carboxymethyl chitosan salts, especially sodium salts, get easily a membrane hydrolysis at dialysis of the solution is also demonstrated in conductance measurements. These properties are remarkable characteristics as polyampholytes of carboxymethyl chitosan.

Studies on the Ammonia Assimilation by Yeast. Part III. Inhibitory Effects of Schort-Chain Fatty Acids. (p. *479~482*)

By Kiyoshi SUGAWARA and Kensuke SHIMURA

(*Laboratory of Biochemistry, Faculty of Agriculture, Tohoku University, Sendai, Japan*)

The inhibitory effects of acetate, formate, butyrate and valerate on the ammonia assimilation in nitrogen starve yeast, *Torula utilis* were studied. The increases in intracellular soluble nitrogen, amino acids, amides and RNA fell with depression of ammonia utilization, and no particular substances did not accumulated at pH 4.8, 30°C. The inhibitory effect was also observed in the consumption of glucose oxidation in relatively high concentration of fatty acids. However, in low concentration, these consumption was enchanced by acetate ($10^{-2}\,\text{M} \sim 5\times10^{-2}\,\text{M}$) and butyrate ($2.5\times10^{-4}\,\text{M} \sim 10\times10^{-4}\,\text{M}$). On the contrary, the ammonia assimilation was supressed under the same condition, and there was no parallelism between the glucose oxidation and the ammonia assimilation. Synthesis of ether soluble materials and total carbohydrate in the cell was also inhibited by the fatty acids except acetate.

Studies on the Alginic Acid Destroying Bacteria. Part I. On the Identification of one Isolated Strain and its Alginase. (p. *483~486*)

By Kazuo YAMAGUCHI

(*Agricultural Faculty of Ibaraki University*)

A strain of alginic acid destroying bacteria was isolated from standing water in the alginic acid manufacturing factory.

This bacterium is short rod, non-spore forming, gram-negative and motile one.

This bacterium is proposed to be designated as *Cloaca cloacae* A-10 from its mycological characteristics according to Kauffmann's classification.

Optimal conditions for the enzyme action are at pH 7.0-7.4 and 35-40°C.

It was recognized that alginase secreted by *Cloaca cloacae* A-10 was not an adaptive enzyme, but a constitutional one.

Studies on the Digestive Action of Proteases from *Aspergillus oryzae*. The Separation and Purification of Proteases and their Digestive Action.

(p. *486~490*)

By Yûkichi MIURA

(*Sankyo Takamine Laboratory*)

Aspergillus oryzae produced at least three kinds of proteases, which were named "alkaline protease", "neutral protease", and "acid protease" distinguished from the difference of their optimum pHs.

They were separated from the submerged culture of this mold or Takadiastase, and each obtained in purified states which were homogeneous on the paper electrophoresis.

Their digestive action to animal and vegetable proteins were investigated.

In general, meat, egg, soy-bean flour, and gluten were well digested by each of them.

Although alkaline protease was the most powerful one, it had a slight activity to uncooked fish, and no activity to egg white.

Egg white contained an inhibitor against the alkaline protease, which was destroyed by the acid protease.

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